The mechanism of antimicrobial action of Electro-Chemically Activated (ECA) water and its healthcare applications

by

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"Whoever undertakes to set himself up as judge in the field of truth and knowledge is shipwrecked by the laughter of the Gods."
Albert Einstein, October 26, 1929, The Saturday Evening Post.

The supreme goal of all theory is to make the irreducible basic elements as simple and as few as possible without having to surrender the adequate representation of a single datum of experience
"Theories should be as simple as possible, but no simpler"
Albert Einstein, 1933

In every true searcher of Nature there is a kind of religious reverence, for he finds it impossible to imagine that he is the first to have thought out the exceedingly delicate threads that connect his perceptions.
Albert Einstein, 1920.

"Everyone who is seriously involved in the pursuit of science becomes convinced that a spirit is manifest in the laws of the Universe-a spirit vastly superior to that of man.... In this way the pursuit of science leads to a religious feeling of a special sort, which is indeed quite different from the religiosity of someone more naïve".
Albert Einstein , January 24, 1936; Einstein Archive 42-601

I don't try to imagine a God; it suffices to stand in awe of the structure of the world, insofar as it allows our inadequate senses to appreciate it.
Albert Einstein , April 16, 1954; Einstein Archive 30-1154

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DECLARATION

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

Signed …………………………………………

This………………………… day of…….…………………….………., 2009

LIST OF ABBREVIATIONS

ACC: Available Chlorine Concentration

ADP: Adenosine Diphosphate

AEW: Acidic Electrolysed Water

AFM: Atomic Force Microscopy

AIR: Airborne Infection Research

AISI: American Iron and Steel Institute

AME: Aminoglycoside Modifying Enzyme

ATCC: American Type Culture Collection

ATP: Adenosine Triphosphate

CAT: Catalase

CDC: US Centres for Disease Control and Prevention

CFU: Colony Forming Unit

CSIR: Council for Scientific and Industrial Research

DNA: Deoxyribonucleic Acid

DBP: Disinfection Bi-Product

DPD: N,N-Diethyl-p-Phenylenediamine.

EAW: Electrochemically Activated Water

ECA: ElectroChemical Activation

EDTA: Ethylenediaminetetraacetic acid

EMF: Electromotive Force

EOW: Electrolysed Oxidising Water

EPA: Environmental Protection Agency

EPS: Extracellular Polymeric Substance

ESBL: Extended Spectrum ß Lactamase

EW: Electrolysed Water

FAC: Free Available Chlorine

FAD: Flavin Adenine Dinucleotide

FAO: Free Available Oxidants

FDA: Food and Drug Administration

FEM: Flow Electrochemical Module

HAA: Haloacetic Acid

HAI: Hospital Acquired Infection

HELP: High Electric Field Pulses

LPS: Lipopolysaccharide

MDR: Multi Drug Resistant

MIC: Minimum Inhibitory Concentration

MMC: Minimum Microcidal Concentration

MRSA: Methicillin Resistant *Staphylococcus aureus*

MSSA: Methicillin Sensitive *Staphylococcus aureus*

MPO: Myeloperoxidase

NAD: Nicotinamide Adenine Dinucleotide

NADH: Nicotinamide Adenine Dinucleotide (Reduced)

NB: Nutrient Broth

NICU: Neonatal Intensive Care Unit

OADC: Oleic acid Albumin Dextrose Catalase

ORP: Oxidation Reduction Potential (=REDOX)

PBDW: Phosphate Buffered Diluent Water

PBS: Phosphate Buffered Saline

PEF: Pulsed Electrical Fields

PMF: Proton Motive Force

PPD: Protein Purified Derivate

RFE: Flow Electrochemical Reactor

RNA: Ribonucleic Acid

ROS: Reactive Oxygen Species

SAMRC: South African Medical Research Council

SEM: Scanning Electron Microscopy

SOD: Superoxide Dismutase

SOW: Super-Oxidized Water

SPM: Scanning Probe Microscopy

STP: Standard Temperature and Pressure

SRB: Sulphite Reducing Bacteria

THM: Trihalomethane

TOX: Total organic halogen

TSA: Tryptone Soy Agar

TSB: Tryptic Soy Broth

TST: Tuberculin Skin Testing

USDA-FSIS: United States Department of Agriculture – Food Safety Inspection Services

USPTO: United States Patent and Trade Mark Office

VRE: Vancomycin Resistant *Enterococcus*

WIPO: World International Patent Organisation

Glossary of ElectroChemically Activated Radical species

Anolyte solution

Cl_2 - Chlorine

Cl^- - Chloride ion

$Cl\cdot$ - Chlorine radical

OCl^- - Hypochlorite anion

$ClO\cdot$ - Hypochorite radical

ClO – Chlorine Oxide

Cl_2O – di–Chlorine oxide

ClO_2 – Chlorine Dioxide

ClO_2^- - Chlorite anion

ClO_3^- - Chlorate anion

ClO_4^- - Perchlorate anion

HClO, HOCl, ClOH – Hypochlorous acid

HOClO – Chlorous acid

$HOClO_2$ – Chloric acid

$HOClO_3$ – Perchloric acid

HCl – Hypochloric acid/hydrochloric acid

$NaCLO_2$ – Sodium chlorite

O_2 – Oxygen

O_3 – Ozone

$O\cdot$ - Oxygen radical

$^1O_2$ – Singlet oxygen

O_2^- - Superoxide radical

$O_2\cdot^-$ - Superoxide anion

O^{2-} - Oxide ion

O_2^{2-} - Peroxide anion

ONO_2^- - Peroxynitrite

H_2O_2 – Hydrogen Peroxide

OH^- - Hydroxyl

HO_2^- - Hydrogen dioxide, hydrogen peroxide anion

$HO\cdot$ / $OH\cdot$ - Hydroxyl radical

$HOO\cdot$ - Hydroperoxyl radical / Perhydroxyl radical

Catholyte solution

$NaOH$ – Sodium Hydroxide

Na – Sodium

H_2O_2 – Hydrogen Peroxide

$H_3^-O_2^-$ - Stable peroxide

O_2^- - Superoxide radical/Dioxide

$O^{2-}{}_2$ – Peroxide anion

HO_2 - Hydrogen dioxide

HO_2^- - Hydrogen dioxide, hydrogen peroxide anion

$HO\cdot_2$ – Hydrogen dioxide radical

H_2 – Molecular Hydrogen

$H\cdot$ - Hydrogen radial

OH^- - Hydroxyl

$OH\cdot$ - Hydroxyl radical

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The mechanism of antimicrobial action of Electro-Chemically Activated (ECA) water and its healthcare applications

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Department : Microbiology and Plant Pathology

Degree : PhD

Summary

The Electrochemical Activation (ECA) of water is introduced as a novel refinement of conventional electrochemical processes and the unique features and attributes are evaluated against the universal principles that have described the electrolytic processes to date. While the novel and patented novel reactor design retains the capacity to generate products common to conventional electrolysis, it also manipulates the properties of the reagent solutions to achieve an anomalous Oxidation-Reduction potential (ORP or REDOX) that cannot be replicated by traditional chemical and physical interventions. As a contemporary development in the field, the technology continues to undergo rigorous assessment and while not all of its theoretical aspects have been exhaustively interrogated, its undisputed biocidal efficacy has been widely established.

Microbial vitality has been shown to be directly dependent upon the confluence of a diverse variety of physical and chemical environmental conditions. Fundamentally important in this regard is the electronic balance or REDOX potential of the microbial environment. The intricate balance of metabolic pathways that maintain cellular integrity underwrites the measures of irritability required for sustained viability. Aside from the direct effects of the conventional electrolysis products, overt electronic disruption of the immediate microbial environment initiates a cascade of secondary and largely independent autocidal molecular events which compromise the fundamental integrity of the microbe and leads to cell death.

The distinctive capacity to impart unique physicochemical attributes to the ECA derived solutions also facilitates the characterisation of the same outside of the conventional physicochemical and gravimetric measures. These adjunct measures display a substantial relationship with the predictability of antimicrobial effect, and the direct relationship between inactivation of a defined microbial bioload and the titratable measures of REDOX capacity have been shown to describe a repeatable benchmark.

The use of ultra-microscopy to investigate the impact of the ECA products on bacterial cell structures has shown this tool to have distinctive merit in the imaging and thus refined description of the consequences of exposure to biocidal solutions.

The distinctive differences of the ECA solutions relative to conventional antibacterial compounds would suggest a heightened suitability for application in conditions where the efficacy of conventional biocidal compounds had been limited. Aeroslisation of the ECA solutions for the decontamination of airspaces challenged with tuberculosis pathogens revealed that despite initial success, further refinements to the application model will be required to meet the unresolved challenges.

The health care benefits associated with the application of the ECA solutions in a medical environment substantiate the merits for the adoption of the technology as a complementary remedy for the management of nosocomial infections. The relative novelty of the technology in the commercial domain will raise questions regarding the potential for resistance development, and it has been proposed that the distinctive mechanism of biocidal action will not contribute to diminished bacterial susceptibility, as it does not reveal any cross- or co-resistance when assessed against multiple antibiotic resistant strains.

These benefits are further reinforced by the capacity to install the technology for both on-site and on-demand availability, and being derived from natural ingredients (salt and water) the ECA solutions are regarded as safe and compatible for general in-contact use. Notwithstanding the multiple benefits that the technology may provide, further assessments into materials compatibility as well as potential by-products formation following environmental exposure are imperative before the unfettered adoption of this technology as a cost-effective, safe and reliable alternative to conventional disinfection can be promoted.

Patents

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Kirkpatrick, R.D. (2008). Beverage manufacture, processing, packaging and dispensing using Electrochemically Activated water. USPTO, US 61/026,960.

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Chapter 1

Introduction and historical development of Electrochemical Activation

1.1 Water: Introduction

Liquid water is the essential biological solvent of all life processes (Ludemann, 1993). It also represents the continuous phase of all living organisms. However despite being the most abundantly occurring inorganic liquid (Savage, 1993), its familiarity and ubiquitous nature has led to it being regarded as a bland, inert liquid, or a mere space filler for living organisms (Lehninger, 1975). Despite this, most biological molecules of a living organism only function in water, and thus it remains fundamental for the optimal functioning of all life processes (Prilutsky and Bakhir 1997).

A most important consequence of the polar nature of water and of its dynamic capacity for hydrogen bonding (Stumm and Morgan, 1996) is that water is a so-called *"universal solvent",* and as such is yet another reason why it is indispensable to life itself. Water it is a highly reactive substance, and its ionization products, hydrogen and hydroxide, are important determinants of the characteristic structure and biological properties of proteins and nucleic acids, as well as the interaction with different organic or inorganic intracellular compounds, membranes, ribosome's and many other cellular components (Lehninger, 1975; Prilutsky and Bakhir, 1997).

1.2 Water: Structure and behavior

As a chemical substance water is unique, and its composition and characteristics have no analogue in nature (Lehninger, 1975). Water is, in fact, considerably more complex than it appears at first sight; an indication of this is the complex configurations displayed by snowflakes. Nevertheless, a very simplified description of water (H_2O) is that it consists of molecules made up of two hydrogen (H) atoms and one oxygen atom (O). Water is capable of existing in a variety of states i.e. crystalline ice (11 variant forms), amorphous ice (non-crystalline), crystalline hydrates (organic and inorganic), liquid water (ordinary, super-cooled and vapour), aqueous solutions (ionic and non-ionic) and as a gaseous state (monomers and

clusters). The strong intermolecular forces in liquid water are caused by the specific distribution of electrons in the water molecule. Each of the two hydrogen atoms shares a pair of electrons with the oxygen atom, this through an overlap of the 1s orbital of the hydrogen atoms with the two hybridized sp3 orbitals of the oxygen atom (Lehninger, 1975, Stumm and Morgan, 1996). This arrangement of electrons in the water molecule imparts its distinctive electrical asymmetry. The hydrogen atoms have an excess of positive charge and the oxygen atom an excess of negative charge. The highly electron negative oxygen atom tends to withdraw the single electrons from the hydrogen atoms leaving the hydrogen nuclei exposed – hence each hydrogen atom has a local positive charge (δ^+) versus the local negative charge (δ^-) in the unshared orbital of the oxygen atom (Fig 1). Thus while the water molecule has no net charge, it still exists as an electric dipole (Lehninger, 1975, Stumm and Morgan, 1996).

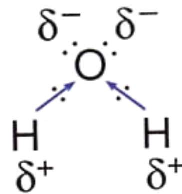


Figure 1. Charge distribution on a water molecule that confers it's the bipolar nature (Schwartz, 1997).

Hydrogen bonding refers to the phenomenon when a unscreened proton attaches to another atom, giving rise to a polarisation of a subsidiary bond much weaker than a covalent bond but stronger than a normal van der Waals force. The two positive protons of the two hydrogen molecules find the negative lone pair electrons of the oxygen and consequently join to produce a co-ordinated cross linked structure of exceptional strength and stability (Fig 2).

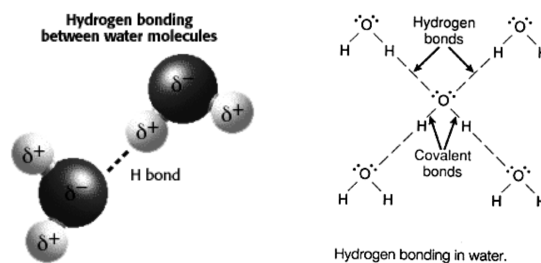


Figure 2. Schematic illustrating the hydrogen bonding associated with the dipolar nature of the constituent atoms (Schwartz, 1997).

When water molecules approach each other, an electrostatic attraction between the partial positive charge of the hydrogen atoms of one water molecule and the partial negative charge of the oxygen atom of the adjacent water molecule arises. The ensuing redistribution of electrical charges gives rise to a complex electrostatic union referred to as a hydrogen bond (Lehninger, 1975; Stryer, 1981).Hydrogen bonds are energetically weak when compared to covalent bonds. Hydrogen bonds in water are estimated to have a bond energy of approximately 18-20 kcal mol^{-1}, compared to the covalent bond energy of 360-400 kcal mol^{-1} between the H-O atoms (Lehninger, 1975; Stumm and Morgan, 1996). The formation of a single hydrogen bond predisposes to the formation of additional hydrogen bonds between adjacent molecules, and facilitates an enhanced association between all solute molecules. This enhancement of the strength of attraction between two molecules arising from the cooperation of several weak bonds is termed cooperativity. This cooperativity of hydrogen bonding is also a characteristic that confers structure to proteins and nucleic acids. This fact together with geometrical specificity and directionality, endows hydrogen bonds with a greater biological advantage over covalent bonds in biomolecular circumstances (Lehninger, 1975).

Water is conventionally regarded as a ‘four-sided’ or tetrahedrally co-ordinated lattice of hydrogen bonded water molecules. However its behaviour under the different states of existence means that there is no uniform dictate which governs its structure. The basic characteristics of water structure are related to the maximisation of the number of hydrogen bonds. This is coupled to the repulsive restrictions that other charged molecules will play in determining the resulting geometries and will directly affect the conventional tetrahedrality (Stryer, 1981; Savage, 1993). Due to the tetrahedral arrangement of electrons around the oxygen molecule, each water molecule is theoretically capable of hydrogen bonding with four neighbouring water molecules. It is this electrostatic attraction that confers the distinctive internal cohesion of liquid water. The conventional tetrahedral cluster of 5 water molecules has been determined to be 0.5 nm in diameter (Lehninger, 1975).

With an increase in the temperature of liquid water, there is a parallel increase in the degree of distortion of the hydrogen bonds away from a linear alignment and results in a heightened degree of instability of association. Thus there is a temperature

dependent and by direct implication, a highly dynamic energy driven association, wherein the hydrogen atoms continuously transform their attractive associations by acting as positively charged bridges between the adjacent negatively charged oxygen atoms. For a given energy state this will result in the formation of "clusters" comprising of up to 13-14 water molecules (Lehninger, 1975).

1.3 Water as a solvent

Water is recognised to be a significantly superior solvent when compared to other liquids. Many crystalline salts and ionic compounds readily dissolve in water, and in the case of brine, the sodium chloride molecules readily dissociate into stable hydrated Na^+ and Cl^- ions through the attraction with the water dipoles. Water also opposes the electrostatic attraction between positive and negative ions, and the high dielectric constant, specifically promotes the dissolution of molecular conjugates such as NaCl. The presence of dissolved solutes in a solution changes the structure and electronic properties of liquid water. When a salt such as sodium chloride is dissolved in water, the Na^+ and Cl^- ions become surrounded by shells of water dipoles (Fig 3).

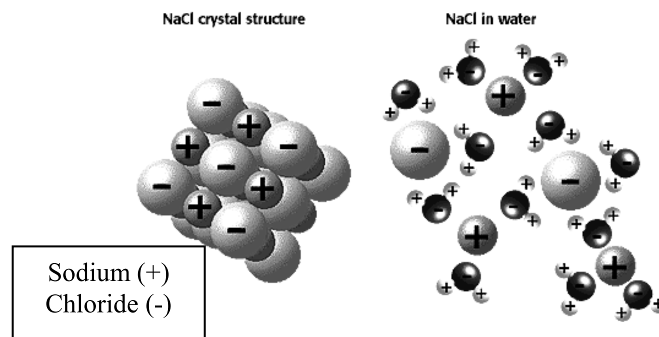


Figure 3. Crystalline manipulations of NaCl in an aqueous solution (Schwartz, 1997)

Within these ion hydrates, the geometry of the water molecules differs substantially from that of the hydrogen bonded water molecules in pure water, and the water molecules become more highly ordered and regular in structure. Dissolved salts display a tendency to disrupt the basic structure of water (Stumm and Morgan, 1996). This has direct thermodynamic implications and the capacity to effect a change in structure is referred to as the 'Colligative Property' of water. Depending on the number of solute molecules per unit volume of solvent, the thermodynamic shifts are

characterized by the depression of freezing point, elevation of boiling point and the depression of the vapour pressure. These solute molecules also confer the property of osmotic pressure (Lehninger, 1975: Zubay *et al.*, 1995).

Thus Oxidation Reduction Potential (ORP or REDOX) values of common (non-activated) chemical solutions are determined by the ratio of reduced (Electron-donor) and oxidised (electron-acceptor) chemical conjugates. If the reduced and oxidised forms of a compound are dissolved in distilled water in definite proportions, a readily quantifiable chemical redox pair is formed (Lehninger, 1975). Coupled to this chemical reaction, when a system of different electrodes i.e. platinum and Silver-Chlorine, are immersed in such a solution without any partition in the inter-electrode space, an electric potential is generated. This electric potential will change in a readily predictable stoichiometric manner when the interrelationships of the various redox–pair components are altered by changing the reagent concentrations within the electrolyte medium (Prilutsky and Bakhir, 1997).

1.4 Energetic status of water

Due to their bipolar nature, the ‘stable’ configuration of water molecules will reflect a balance between the energies of the ubiquitous van der Waals forces of attraction (8-20 kcal mol^{-1}) and those of hydrogen bonding (18.8 kcal mol^{-1}). Since this relationship is energetically biased towards the van der Waals forces, the standard structure of water will thus comprise reduced levels of hydrogen bonded clustering (Wyllie, 1965).

Water has a higher melting point, boiling point, heat of vaporization, heat of fusion, and surface tension than any of its comparable hydrides (H_2S, NH_3). All of these properties are a consequence of the power of attraction between the molecules in the liquid water medium and is a reflection of the state of relatively high internal cohesion. The heat of vaporization is a direct measure of the energy required to overcome the attractive forces between adjacent molecules in the liquid phase so that individual molecules can escape and enter the gaseous phase (Lehninger, 1975). However, the intrinsic energy of molecular association due to van der Waals forces becomes insignificant when substantial external energetic manipulations are applied

to destruct these aggregates of water molecules (Bakhir, 1999). Thus specific electric or equivalent electromagnetic interventions aimed at manipulating this 'background' energy of association, produce significant deviations in the behaviour of the water molecules and their tendency to align in predictable geometric patterns.

Under low magnetic simulations, an increase in the presence of water mono-molecules has been shown to occur with a concurrent increase in tetrahedrality of alignment and clathrate formation (Kotz and Purcell, 1991). Additionally it has been shown that within a high electric field (5 x 10^{9} Vm^{-1}), the structures of association alter to the extent that conventional freezing patterns at low temperatures are inhibited (Wyllie, 1965).

Charge and energy may be rapidly transferred between adjacent water molecules and the effects of electronic excitation can be readily described with studies of cluster disassociation and molecular distortion. This has been attributed to the short half-life of the hydrogen bonds i.e. 100 picoseconds or 10^{-11}sec (Lehninger, 1975; Stumm and Morgan, 1996). Any water structure with a predominance of hydrogen bonds will impede chemical reactions due to an increase in viscosity, a reduction in diffusivity and a reduction in the active participation by water molecules. Thus any factor that reduces the quantity of hydrogen bonding as well as the hydrogen bond strength such as electric fields will encourage the overall reactivity of the molecular water (Chaplin, 2007).

'Transport' is described as the movement of charge, energy or material in a steady but spatially homogenous situation. The transport of charge through a given structural configuration of water, involves the rapid movement of H_3O^+ and OH^- ions between neighbouring water molecules by means of proton exchange. Protons move rapidly through a coherent sequence of hydrogen bonds by a mechanism of relay charge transfer. This movement of charge creates polarised tracks or energetic defects which repair by means of a relaxation process. Water clusters, even under random arrangements have equal hydrogen bonding in all directions. Contact with metallic electrodes at even low voltages has a distinctive effect on the orientation of water molecules and the positioning of ions (Chaplin, 2007).

In low density electromagnetic fields, the translational and rotational motions of the water molecules are reduced, and the reduction in the van der Waals attractive forces strengthen the hydrogen bonds resulting in the water molecules becoming more tightly bound. This in turn influences the solubility of the water and results in an increase in the concentration of dissolved gases and hydrophobic molecules at the contact surfaces. The increase in solution reactivity is ascribed to the evolution of singlet oxygen ($^1O_2$), free radical formation eg. OH·, and specific phase changes as evidenced by the production of flattish surface nanocavities or nanobubbles (Chaplin, 2007).

These changes result in significantly durable effects, and substantiate claims of a so-called 'memory effect'. The manipulation of water structure by electric fields to produce metastable clathrates has also been reported, as well as the paradoxical increase in surface tension due to the relative increase in hydrogen bonding (Zenin, 1999; Chaplin, 2007). Due to the fine balance between the conflicting hydrogen bonding and non-bonded interactions within water clusters, any weakening of the van der Waals forces leads to a strengthening of the hydrogen bonding with greater cyclic hydrogen bonded clustering. This phenomenon has been supported by the increase in the melting point of the water which arises from a greater ordering and lower entropy of the manipulated water in the magnetic field (Chaplin, 2007).

1.5 Electrolysis

1.5.1. History of Electrolysis

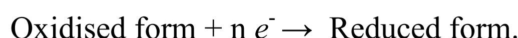
In 1832, Michael Faraday introduced the principle of electrolysis and postulated the laws that govern the passage of electric current through an aqueous salt solution (Shirahata *et al.*, 1997). This law states that the weight of a given element liberated at an electrode is directly proportional to the quantity of electricity that is passed through the solution (Kotz and Purcell, 1991). The first patent for the electrolytic manufacture of chlorine was submitted by Charles Watt in 1851, and the first commercial production of chlorine by electrolysis was commissioned in Germany in 1890 (White, 1992).

1.5.2 Energy of Electrolysis

While conventional electrochemistry describes the ability to generate an electric current from chemical reactions, electrolysis describes the use of an electric current to bring about a chemical change in the electrolyte (Kotz and Purcell, 1991).

In terms of basic description, an electrolytic cell consists of a pair of inert electrodes in contact with a solution of brine in a liquid state, where the ions are dissociated and free to move. The connection of the electrodes to an external source of electrical current creates an ‘electron pump’, which causes one of the electrodes to become negatively charged. Monovalent cationic sodium ions are attracted to this negative electrode, and become reduced when electrons from the electrode are accepted. This reducing action at the electrode results in it becoming the cathode.

The current source simultaneously draws electrons from the other electrode giving it a nett positive electrical charge. Chloride anions are attracted to this electrode where they surrender electrons to become oxidised. This reaction results in this electrode becoming the anode (Kotz and Purcell, 1991). Oxidation occurs when an atom, ion or molecule releases electrons and becomes oxidised. Conversely reduction occurs when an atom, ion or molecule gains electrons, thus becoming reduced. Thermodynamically there are no free electrons,and thus every oxidation reaction must be accompanied by a corresponding reduction (Stumm and Morgan, 1996). Thus a reducing agent donates electrons, whereas an oxidising agent receives or scavenges electrons. The reaction can be summarised as follows:



$$\text{Oxidised form} + n\, e^{-} \rightarrow \text{Reduced form.}$$

Electrons generated at the site of oxidation, the anode, are driven or pushed towards the cathode by an electromotive force or *emf*. This force is directly proportional to the difference in energy between the two electrodes and also reflects upon the reactivity and concentration of the electrolyte solution (Lehninger, 1975).

For a given energy (charge) differential between the two electrodes in an electrolysis system, there is an equivalent potential accorded to the electrolytic cell which is

termed the cell potential or E. Under standard conditions i.e. concentration and pressure, this potential is referred to as the 'standard cell potential' and is designated as E^o. Thus the potential produced by an electrolytic cell is the sum of all potentials of the oxidising and reducing half-reactions in the system.

The standard free energy (G^o) of an oxidation-reduction reaction is proportional to the cell potential under standard conditions and the relationship is governed by the following equation:

$$\Delta G^o = - nFE^o$$

where n refers to the number of electrons transferred between oxidising and reducing agents and F is the Faraday constant (9.64853×10^4 coulombs/mole of electrons).

Additionally, this change in free energy (ΔG^o) in an oxidation-reduction reaction under standard conditions is directly proportional to the cell potential, and under conditions where the redox reaction of the cell is balanced, the relationship can be formulated to describe the Nernst equation, namely:

$$E = E^o - {}^{RT}/_{nF} \ln Q$$

where R is the gas constant, T is the temperature (Kelvin), Q is the reaction quotient (${}^{[oxidants]}/_{[reductants]}$), F is the Faraday constant and n, is the number of electrons transferred between the oxidising and reducing agents in a balanced redox equation (Stumm and Morgan, 1996).

The Nernst equation thus permits the theoretical calculation of the voltage produced by an electrolytic cell or conversely describes the concentrations of oxidising or reducing agents present for a given energy input. This equates to the REDOX potential that is produced when the voltage applied across the cell is known. From a biological perspective, the Nernst equation describes the relationship between the voltage across a semi-permeable membrane and the ion concentration in the compartments on either side of the membrane. The equation provides a simple method for testing the status of the electronic equilibrium of solutes across the membrane system (Sperelakis, 1995). The number of moles of electrons consumed or

produced in a redox reaction is obtained by measuring the current flowing in the external electric circuit at a given time.

It is also possible to quantify the charge in terms of the number of electrons that comprise the electrochemical reaction. This equates to the charge carried by one mole of electrons and equals 1 Faraday (F) where:

$$1\ F = (1.602177 \times 10^{-19}\ \text{coulombs/electron})(6.02214 \times 10^{23}\ \text{electrons/mol})$$
$$= 9.64853 \times 10^{4}\ \text{coulombs/mol (= Faraday's constant).}$$

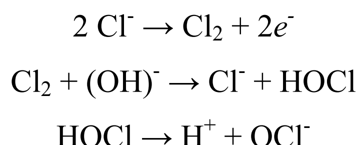
Based on the relationship between charge, current and time, the charge can be calculated by multiplying the current and the time of the reaction. By combining the chemistry of the redox reactions with the calculated energy input of the system, one is able to establish the measure of the electrochemical activation of the electrolytes in the system (Kotz & Purcell, 1991). Thus from a theoretical perspective, there is a direct relationship between the relative concentrations of the oxidising or reducing agents that are produced when the voltage applied across the cell is known and when the redox reaction is balanced.

1.5.3 Chemistry of Electrolysis

Conventional electrochemistry is founded on the following reaction.

$$NaCl + H_2O + \text{electric current} \rightarrow NaOH + \tfrac{1}{2}\,Cl_2 + \tfrac{1}{2}\,H_2$$

The reaction of the brine solution at the Anode would comprise:



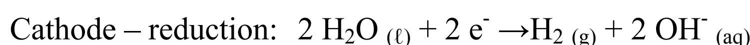
$$2\ Cl^- \rightarrow Cl_2 + 2e^-$$
$$Cl_2 + (OH)^- \rightarrow Cl^- + HOCl$$
$$HOCl \rightarrow H^+ + OCl^-$$

Correspondingly and simultaneously, the reaction at the Cathode would be:

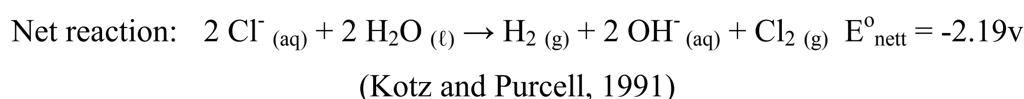
$$2\ H^+ + 2\ OH^- + 2\ e^- \rightarrow H_2 + 2\ OH^-\ \text{(White, 1992).}$$

However it has long been shown under practical conditions, that this premise does not hold true and the best description of the chemistry that occurs during electrolysis of aqueous NaCl is the following:

$$\text{Anode – oxidation:} \quad 2\,Cl^-_{(aq)} \rightarrow Cl_{2\,(g)} + 2\,e^-$$



$$\text{Cathode – reduction:} \quad 2\,H_2O_{(\ell)} + 2\,e^- \rightarrow H_{2\,(g)} + 2\,OH^-_{(aq)}$$

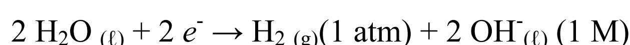


$$\text{Net reaction:} \quad 2\,Cl^-_{(aq)} + 2\,H_2O_{(\ell)} \rightarrow H_{2\,(g)} + 2\,OH^-_{(aq)} + Cl_{2\,(g)} \quad E^o_{nett} = -2.19v$$

(Kotz and Purcell, 1991)

It is imperative to acknowledge that the ions of sodium and chloride in an electrolytic reaction do not act in isolation, and that they represent only one dimension of the electrolyte solution. Thus instead of dismissing the role of the water as a mere background filler or inert solvent, the evaluation of the composite electrolytic reaction must encompass the decisive role played by the dissociation of the individual water molecules and the constituent ions when considering definitive calculations of electrolytic reactions. The anomalous behaviour of the ions in the electrolysis cell can be explained by the mismatch between the conventional quantitative calculations and the actual reaction speed or kinetics of the various reactions that occur within the system.

This anomalous behaviour has been termed the 'over-potential' and is particularly well demonstrated with the electrolytic reduction of water:



$$2\,H_2O_{(\ell)} + 2\,e^- \rightarrow H_{2\,(g)}(1\text{ atm}) + 2\,OH^-_{(\ell)}\,(1\text{ M})$$

This reaction has a calculated potential of $E^o = -0.83V$, however the measured reaction occurs at an even more negative potential value ~ -1.4V (Kotz and Purcell, 1991). Recognition of this anomaly contributes substantially to the understanding of the phenomenon of the Electro-Chemical Activation (ECA) of water as advanced in the first reports by the inventor (Prilutsky and Bakhir, 1997; Bakhir 1999).

1.5.4 Conventional Brine electrolysis

The ideal electrochemical cell consists of an Anode and a cathode and a separating partition where the separator permits selective migration of ions between the chambers (Kotz and Purcell, 1991).

Commercially chlorine is produced by the electrolysis of a concentrated sodium chloride solution. When dissolved in water, the dissociated chloride anions are oxidised at the anode to form chlorine gas, while the water molecules are reduced at the cathode to form hydroxyl anions and hydrogen gas. The sodium ions in solution react with the hydroxyl ions to produce sodium hydroxide (Bakhir and Zadoroznhy, 1999[a]; Bommaraju *et al*., 2007).

Chlorine is produced electrolytically using three basic types of reactor cells. These comprise the mercury type, diaphragm type and membrane based cells. The main difference between the different reactor types in the manner by which the chlorine gas and the sodium hydroxide are prevented from admixing in order to ensure the generation of products of high purity (Bommaraju *et al*., 2007). In mercury based cells there is no physical separator, and the electrolytic process requires that the mercury itself, acts as a separator. Conversely, in diaphragm cells, the partitioning of the electrolysis products is due to a material separator which may be either asbestos or an equivalent polymer-modified coating of the cathode. In membrane type cells, the separating partition is usually made of an ion-exchange membrane (Fig 4) (Curlin et al, 1991). While all earlier cells made use of carbon based anodes, modern anodes are made with standardised and dimensionally stable electrodes which are titanium based and coated with a layer of specific platinum group metal (PGM) oxides designed to optimally catalyse the electrolytic reaction and limit corrosion (Diao *et al.*, 2004). The cathode is typically steel in diaphragm cells, nickel in membrane cells, and mercury in mercury cells (Curlin *et al*., 1991).

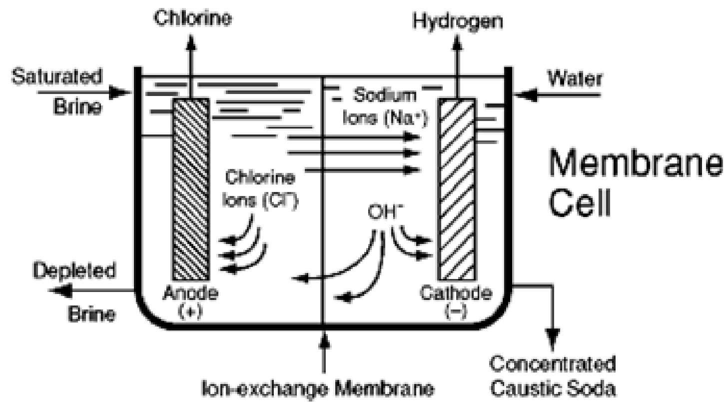


Figure 4. Schematic of a conventional membrane based electrolytic cell (Curlin *et al.*, 1991).

In a membrane type cell, the acid based ion-exchange membrane is sandwiched between the anode and cathode plates. Electrolysis of the saturated brine solution fed into the anodal compartment results in the evolution of chlorine gas at the anode, while the cationic sodium ions migrate through the membrane into the cathodal compartment (Curlin *et al.*, 1991). In contrast to the diaphragm type cells, only water and sodium ions pass through the membrane, and the unreacted sodium chloride remains in the anolyte. The sodium ions within the cathode chamber react with the hydroxyl ions generated by the dissociation of the water and results in the formation of sodium hydroxide, caustic soda or "caustic" which is extracted from the cathodal chamber. As a direct consequence of the water dissociation, the solution becomes saturated with hydrogen gas.

A further variation of basic cell type is employed for Sodium hypochlorite or "bleach" production. These reactor cells electrolyse dilute brine solutions and comprise an anode and cathode without a separating diaphragm or membrane (Bommaraju *et al.*, 2007). The anodic and cathodic reactions are equivalent to that of a chlor-alkali cell and the main difference is that the pH of the electrolyte in the cell is maintained in the range of 10 to 12. Under these conditions, the chlorine generated at the anode reacts with sodium hydroxide to form sodium hypochlorite (NaOCl). This cell type is functionally inefficient in that only low concentrations of bleach are produced as the hypochlorite is readily reduced at the cathode to reform into chloride. Additionally, when the electrolyte becomes acidic, the hypochlorite can also react further to form chlorate (Bommaraju *et al.*, 2007).

1.5.5 Electrochemical Activation (ECA) of water

1.5.5.1 History of Electro-Chemical Activation

Thirty years prior to the publication by Michael Faraday of the laws of electrolysis (1832), Petrov described the evolution of electrolytic gases from a high powered galvanic cell. In addition, by introducing a porous diaphragm between the electrodes, he was able to harvest separate products from the anodal and cathodal compartments, and termed these solutions anolyte and catholyte respectively (Prilutsky and Bakhir, 1997).

It is reported that between 1960 and 1973, Korovin conducted a variety of experiments into the behaviour of water in an electrostatic field and reported on a series of effects that had previously not been described. He referred to a process of "water energisation" wherein "charged water was able to stimulate the activity of living matter and in this manner enhanced the resistance to disease" (Prilutsky and Bakhir, 1997). In Japan, Suwa was reported to have designed a diaphragm type electrolyser capable of producing separate Anolyte and Catholyte solutions from water as far back as 1952. These reports led to the creation of the terms "Live and Dead" water, which regretably eventuated in a series of spurious and technologically unsubstantiated commercial fads (Prilutsky and Bakhir, 1997).

In 1972, Bakhir formally introduced the process of "ElectroChemical Activation (ECA) of water", where anolyte and catholyte solutions generated from water of low-mineralisation, were found to display physicochemical parameters and reactivities which were substantially distinct from the models that were governed by the classical laws of electrolysis. These anomalous findings arose from studies conducted into the use of electrochemical methods to modify the properties of drilling solutions for use in oil and gas exploration whilst working at the Tashkent Scientific Research Institute of Natural gas, USSR Ministry of Gas industry (Leonov, 1997).

The term 'Electro-Chemical Activation' was officially coined by Vitold Bakhir in 1975 (Tomolov, 2002).

1.5.5.2 Principles of ElectroChemical Activation (ECA) of water

"There is no progress without paradox" - *Niels Bohr* (Drost-Hansen, 1965).

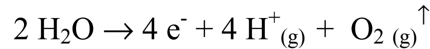
In contrast to conventional electrochemical systems where chemical reactions always take place at the electrode surfaces, and where the chemical composition of the electrolyte in direct contact with the anode and cathode will be subject to predictable alterations, the objective of the Electro-Chemical activation (ECA) of water is to subject the entire volume of liquid in the inter-electrode space to an electric field of the maximum possible intensity, with the minimum chemical exposure possible and with a minimum of heat emission.

The primary limitation of this approach is that the maximum electro-physical effect can only be ensured in the immediate vicinity of the electrode surface i.e the electric-double-layer (EDL) (Bakhir, 1999) The EDL is comprised of one layer attached to the electrode surface and the other which is distributed diffusively in the contact liquid (Stumm and Morgan, 1996). Vicinal water refers to interfacial water i.e. that which is in contact with a solid interface, the properties of which differ from the adjacent bulk water due to the structural differences induced by the proximity to the (electrode) surface. These interfacial anomalies fall outside of established physicochemical ranges, and have historically been described in terms of variations in the temperature of the electrolyte at the electrode surface. These anomalous changes to the properties of vicinal water are fundamentally independent of ion-dipole or dipole-dipole interactions, and are highly energetic and exist regardless of the specific surface interactions (Drost-Hansen, 1965).

These anomalous modifications to the water structure decay progressively with increasing distance from the electrode surface, and have a characteristic decay length of the order of one to a few nanometers i.e. 4-10 water molecule diameters (Drost-Hansen, 1965). Thus the capacity to effect a substantive energetic change to the properties of the water volume in the inter-electrode space is constrained by the magnitude of the Electric-Double-Layer or the volume of vicinal water immediately adjacent to the electrode and the surface charge density or electric potential at the surface (Stumm and Morgan, 1996).

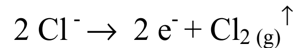
During the ElectroChemical Activation (ECA) of a dilute brine based solution, the following changes are postulated to occur when contrasted against conventional electrochemical models.

Reaction at the Anodal surface:



$$2\,H_2O \rightarrow 4\,e^- + 4\,H^+_{(g)} + O_{2\,(g)}\uparrow$$

The Anolyte would be acidic and oxygen gas would be evolved.



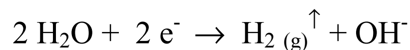
$$2\,Cl^- \rightarrow 2\,e^- + Cl_{2\,(g)}\uparrow$$

Chlorine gas would evolve, and a number of highly active oxidants would be formed.

$$Cl_2O,\ ClO_2,\ ClO^-,\ HClO,\ Cl\cdot,\ O_2,\ O_3,\ HO_2$$

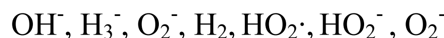
The resultant oxidant solution has a pH of <2 and an ORP of ~1150 mV. It is highly oxidising with potent biocidal properties.

The corresponding electrolysis of brine at the Cathode would be:



$$2\,H_2O + 2\,e^- \rightarrow H_{2\,(g)}\uparrow + OH^-$$

Hydrogen gas would be evolved, the solution would become alkaline,
and a number of highly active reductants would be produced.



$$OH^-,\ H_3^-,\ O_2^-,\ H_2,\ HO_2\cdot,\ HO_2^-,\ O_2^-$$

The resultant catholyte solution has a high pH of > 11.5 and a REDOX of ~ -800mV. It is a highly reducing solution with high adsorption and catalytic capabilities, as well as having surfactant and enhanced wetting properties (Bakhir, 1999). This reaction scheme concurs with the report which details an abundance of dissolved hydrogen in the alkaline cathodal solutions, and a predominance of dissolved oxygen in the acidic anodal water (Shirahata, *et al.*, 1997, Hsu, 2005).

Thus the synthesis of ECA solutions are proposed to only be possible where the unipolar electrochemical exposure of electrolyte solutions is combined with the passage of continuous microvolumes of electrolyte solution through a high voltage field of the electric double layer at the electrode surface (Leonov, 1997). To this end, distinctive electrochemical reactor designs have proven necessary to achieve this objective and these have been refined to optimise the electrolytic dissociation of the water molecule over that of conventional electrochemical processes.

The 'classic' ECA cell comprises a co-axial alignment of electrodes, and the ratio of the relative dimensions of the electrodes ensures an equivalent average electrolyte flow velocity through each partitioned chamber. This unique design facilitates the optimal contact of the maximum possible number of electrolyte microvolumes within the EDL at the electrode surface. The formation of the 'new' highly-reduced or highly-oxidised compounds, free radicals and other reactive molecules occurs within the EDL where the gradient of the electrical field will achieve a potential difference of several million volts per centimetre measure of distance from the electrode surface (Prilutsky and Bakhir, 1997). The qualified presence of these short-lived, energy rich intermediate products with high bacteriocidal capability has been reported by Diao *et al.* (2004).

The historical inability to produce a repeatable unipolar electroactivation outcome, as well as the difficulty to quantify the degree of activation from a purely theoretical perspective, has given rise to the intermediary term of "activation contribution". While incomplete, it serves to define the difference between the physical and chemical parameters of the activated solution immediately after electrochemical exposure, from that which follows the cessation of the relaxation process (Bakhir, 1999). The key to understanding the nature of the ECA process and the creation of transient "metastable" states within the dilute salt solutions, are the changes that arise direct as a result of this unipolar electrochemical exposure.

The phenomenon of electrochemical activation was originally advanced as a result of studying the reactional properties of potable drinking water when subjected to activation in a diaphragm type electrochemical reactor. Given that water does not exist in an ideally pure state in nature, the intrinsic mineralisation may vary considerably both in terms of quality and quantity. Additionally, gases such as oxygen, hydrogen and nitrogen are also dissolved in water. The properties of the water that are produced by Electrochemical Activation are directly dependent upon the composition of the mineral salts of the source water, the type and regimen of electrochemical exposure, and more specifically the design of the electrochemical reactor unit.

Cathodic treatment results in the electrolyte water acquiring an alkaline reactivity due to the dissolved salts which dissociate to form hydroxides. As a consequence, the REDOX decreases, the surface tension falls, the content of dissolved oxygen and nitrogen is reduced, and the concentration of the hydrogen and hydroxyl groups increases. The electric conductivity decreases primarily due to the transformation of heavy metals which precipitate out of solution as insoluble hydroxides (Prilutsky and Bakhir, 1997). Conversely, anodic treatment results in a marked acidity and an increase in REDOX due to the formation of both stable and unstable acids. These include sulphuric, hydrochloric, hypochlorous and persulphuric acids, as well as hydrogen peroxide, peroxosulphates, peroxocarbonates, chloroxy-compounds and their intermediate compounds. In addition, there is a corresponding decrease in the surface tension and an increase in electrical conductivity of the solutions (Prilutsky and Bakhir, 1997; Bakhir, 1999, Hsu, 2005).

Since the degree of water dissociation is negligible, the activity of hydrogen ions in water is thus equivalent to their concentration in solution. This concurs with the anomalous behaviour of dilute electrolyte solutions during electrolysis, wherein the water molecules themselves are acknowledged to contribute to the reactive 'solute' component of a dilute electrolyte solution (Bakhir, 1999).

The REDOX is a measure of the behaviour of electrons or "electron pressure" in a water solution (Stumm and Morgan, 1996). It is measured by a high-ohmic millivoltmeter and accurate assessment requires that a pair of electrodes be used, one of which is the reference electrode i.e. silver chloride (AgCl), while the other is the measuring electrode, which is made from a platinum group metal. There is a distinctive relationship between REDOX and pH, wherein a change in the pH of drinking water by 1 unit by the addition of either sodium hydroxide or hydrochloric acid produces a corresponding change in the REDOX of approximately 59mV i.e. REDOX increases when pH falls and decreases when pH rises (Bakhir, 1999).The exaggerated shifts in REDOX during electrochemical activation of ultrapure water confirm the distinctive differences between theoretical acid-base chemistry and the shifts associated with electrolytic reactions. Nernst also reported on these anomalous reactions where it was found that lower electron activity was associated with an increased REDOX, and where a higher electron potential led to a reduced REDOX

(Bakhir, 1999). It is thus evident that both Anolyte and Catholyte manifest substantial relaxation alterations in terms of REDOX relative to that of the changes observed in the classical chemical models. In the latter, there is near complete parity between the measured and the theoretically calculated values. Conversely, the relaxation changes following electroactivation amounted to deviations of 43 and 100% for the Anolyte and Catholyte solutions respectively following ECA treatment (Fig 5).

				pH - 7.3 ORP: +270mV	1	Drinking Water		
		ANOLYTE		CATHOLYTE		HCl		NaOH
Immediate		pH: 3.03 ORP: + 1040mV ORPt*: + 545mV	2	pH: 11.8 ORP: -830mV ORPt:+18mV		pH: 3.2 ORP: +595mV ORPt +545mV	3	pH: 11.8 ORP:+25mV ORPt: + 18mV
24 hours 1 Day		pH: 3.1 ORP: +1010mV	4	pH: 10.2 ORP: -650mV		pH: 3.01 ORP: 590mV		pH: 11.7 ORP: +20mV
168 hours 7 Days		pH: 3.3 ORP: + 590mV	5	pH: 9.5 ORP: -0mV		pH: 3.02 ORP: +585mV		pH: 11.8 ORP: +20mV
Difference		pH: 0.27 ORP: 450mV	6	pH: 2.3 ORP: 830mV		pH: 0.18 ORP: 10mV		pH: 0 ORP: 5mV

Legend: ORP (REDOX) – Oxidation Reduction Potential, 1 –raw tap water; 2 - freshly generated anolyte and catholyte of dilute saline and tap water; 3 – tap water buffered by hydrochloric acid and sodium hydroxide respectively as direct analogs of electrochemically activated water (control); ORPt* - theoretical redox potential calculated from the Nernst equation; 4 – solution parameters after 24 hours, 5 – solution parameters after 7 days, 6 – changes in parameters over 7 days. (adapted from Bakhir, 1999).

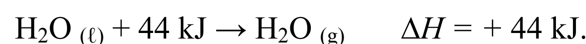
Figure 5. Changes in physicochemical parameters between electrochemically activated brine solutions and their equivalent chemical analogs over time.

When oxygen is bubbled through a dilute hydrochloric acid and water solution at a rate of 100 litres of gas per litre of solution, an increase in REDOX of up to 100mV has been recorded. Conversely, hydrogen gas bubbled through a sodium hydroxide solution, resulted in the generation of a reducing REDOX of –350mV from an initial source solution of 0mV (Chaplin, 2007). This fundamental experiment substantiates the hypothesis that the development of the mismatched pH and REDOX values that

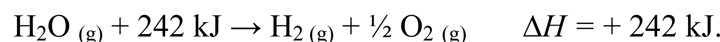
are integral to the ECA process are not mediated by conventional chemical means alone. It is also important to note that increased mineralisation of the source water reduces the magnitude of the relaxation of the pH and REDOX parameters. This phenomenon is confirmed by Hsu (2003, 2005) who reported that high concentrations of salt in the brine feedstock solution resulted in reduced REDOX levels relative to that of dilute brine solutions. The optimum relaxation phenomenon is generated with a brine solution of between 0,1 and 1g/l NaCl, and the amplitude of relaxation deviation decreases when the degree of mineralisation falls below 0,1g/l or rises above 1g/l (Prilutsky and Bakhir, 1997; Bakhir, 1999).

1.5.5.3 Relaxation

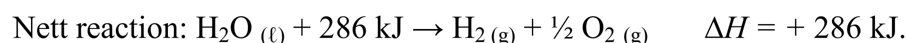
According to the classic thermodynamic definition, relaxation is the gradual transition of a system from an unbalanced state arising from the direct effects of external factors, to that of a state of thermodynamic equilibrium. Relaxation is an irreversible process, and due to the thermodynamic laws governing Entropy and Enthalpy, these changes should be associated with the dissipation of the energy as heat (Tomolov, 2002). Under conventional physico-chemical considerations, heating results in the destruction of water aggregates with a direct increase in the relative concentration of water mono-molecules as well as a solution which displays substantially enhanced chemically reactivity. Heat transferred into or out of a system at constant pressure is defined as Enthalpy. The change in energy of a reaction has been determined to be directly proportional to the overall quantity of reagents present. Hence the energy required to produce the change of phase of water has been detailed as follows:



$$H_2O_{(\ell)} + 44\text{ kJ} \rightarrow H_2O_{(g)} \qquad \Delta H = +\,44\text{ kJ.}$$



$$H_2O_{(g)} + 242\text{ kJ} \rightarrow H_{2\,(g)} + \tfrac{1}{2}\,O_{2\,(g)} \qquad \Delta H = +\,242\text{ kJ.}$$



$$\text{Nett reaction: } H_2O_{(\ell)} + 286\text{ kJ} \rightarrow H_{2\,(g)} + \tfrac{1}{2}\,O_{2\,(g)} \qquad \Delta H = +\,286\text{ kJ.}$$

Thus 286 kJ are required for the endothermic decomposition of 1 mol of liquid water to its gaseous elements (Kotz and Purcell, 1991), and this equates to the energy input

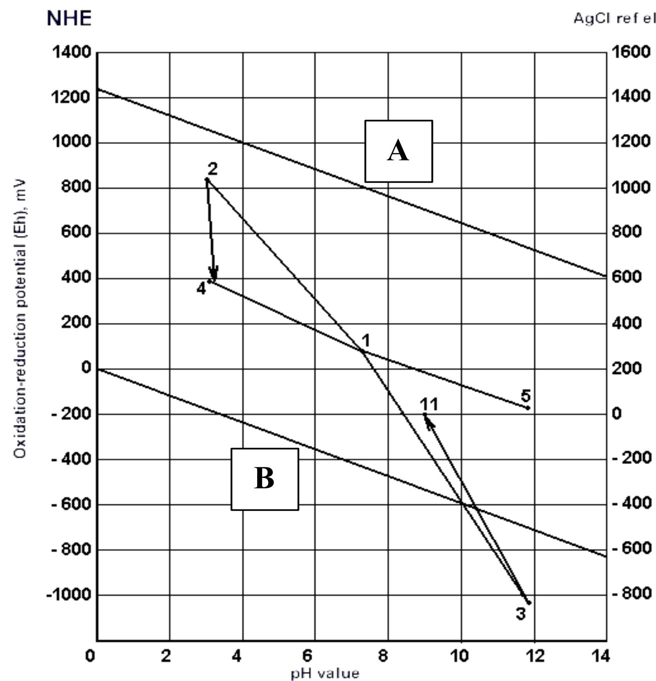
required for the production of steam as would be relied upon for microbial inactivation.

The non-reagent based alteration of the properties of water wherein it acquires anomalous reactional abilities and attributes has been described as "the decomposition of water" with electricity, and results in the physico-chemical modification of the water medium. The term for this change has been coined as 'water de-structurisation' (Bakhir, 1999).

From an energetic perspective, the ECA process has been described as a "series of electrochemical and electro-physical impacts upon water molecules, ions and molecules of the dissolved solutes, under conditions of minimum heat emission, wherein the area of volume charge near the electrode surface of an electrochemical system, results in a non-equilibrium transfer of charge across the electrode: electrolyte interface" (Bakhir, 1999).

Equilibrium is achieved by two processes. The first stage equilibrium is attained only within the micro-volumes of water that comprise the activated solution. The second stage equilibrium is described by a slower relaxation, wherein the physical and chemical parameters reach stationary values which are largely determined by the conditions of equilibrium associated with a standard environment (i.e. STP). This relaxation is directly proportional to the size of the system i.e. the volume of electrochemically activated water, and the slower second phase relaxation processes will encompass such phenomena such as changes in viscosity, diffusion, heat conduction, electrical conductivity, catalytic activity, Oxidation-reduction balance, pH and surface tension (Bakhir, 1999).

A co-ordinate plot of ORP against pH of a standard acid-base manipulation of ORP versus an ECA induced shift highlights the exaggerated range of ORP values which can be achieved with electroactivation (Fig 6).



Legend: A – Upper range of thermodynamic stability of water, B – Lower range thermodynamic stability of water, 1→2 = Anodal activation, 1→4 = water buffered with acid, 1→3 = Cathodal activation, 1→5 = water buffered with alkali, 2→4 = Anolyte relaxation, 3→11= Catholyte relaxation.

Figure 6. Co-ordinate plots of the pH and REDOX values of water as a result of both chemical and electrochemical manipulation of their physicochemical properties and the changes as a result of relaxation (Bakhir, 1999).

Thus it is has been shown that ECA solutions are neither stable nor constant over time, and it is proposed that during “relaxation”, that the reactive properties of the anolyte and catholyte solutions will revert spontaneously with a substantial change in energetic state to the corresponding physico-chemical state of their theoretical chemical analogues (Tomolov, 2002).

The similarity between measured and theortical REDOX values of solutions generated by a plate based reactor as reported by Liao *et al.*, (2007) further substantiates the differences between the latter and the coaxial based FEM design (fig 8), and reaffirms the contention that not all electrolysed oxidizing solutions are the same (Sampson and Muir, 2002)

1.5.5.4 ECA Reactor design

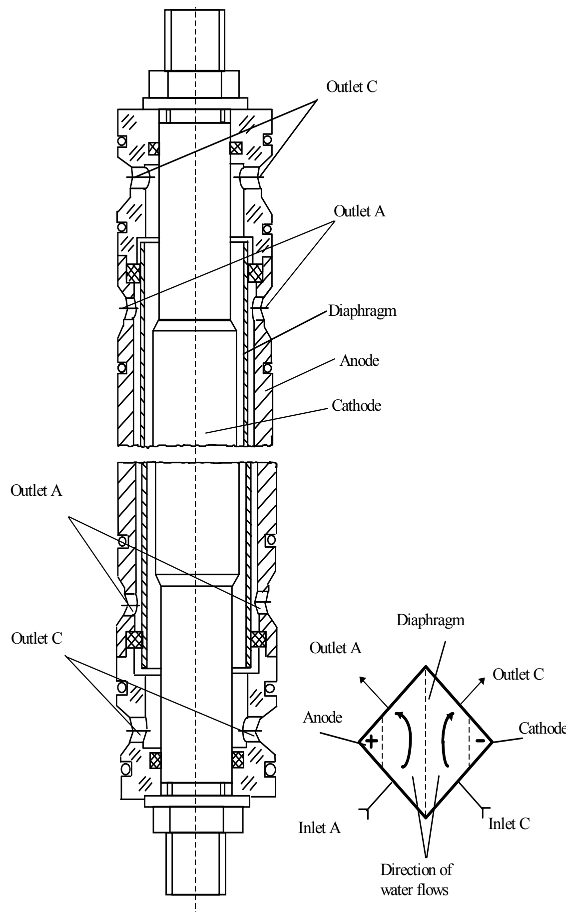
As opposed to conventional electrolytic cells which are designed for selective and concentrated chemical production, the ECA technology is based upon the generation of meta-stable, aqueous based, anolyte and catholyte solutions which display anomalous physico-chemical and catalytic activity. These solutions can only be considered to be in an Electrochemically activated state when the magnitude of the anomalous properties, primarily exaggerated Oxidation-Reduction Potential (REDOX/ORP), are capable of spontaneous reversion to the classical thermodynamic equilibrium which characterise the equivalent, non-activated reagent solutions. The functional integrity and efficiency of the ECA technology thus depends on the consistent unipolar electrolysis of brine solutions of specifically low mineralization. The pure products of ECA electrolysis are not commercially available as distinct chemical reagents, and in the process of the "electrolytic decomposition of water", molecules and compounds are formed which don't exist outside of the electrochemically activated water (Bakhir and Zadorozhny, 1999[b]).

The properties of the electrochemically activated anolyte and catholyte solutions are dependent upon a number of factors which include the solution flow rate through the reactor, the current applied, temperature, the degree of recirculation of solution between the cathodal and anodal chambers, and fundamentally, the degree of mineralisation of the feed water itself. Water of varying, but low degrees of mineralisation is passed through a specifically configured electrochemical cell known as a Flow Electrochemical Module (FEM) or Flow Electrochemical Reactor (RFE). The ECA cell is typically 210mm long with a diameter of 16mm, and comprises a co-axial alignment of the electrodes. It is comprised of a central rod or anode (positive electrode) which is manufactured from titanium and coated with a variety of platinum group metal oxides including ruthenium and iridium (Fig 8) (Bakhir *et al*., 1999; Zinkevich *et al*., 2000)

The anode is inserted into an outer coaxial tube or sleeve which acts as the cathode (negative electrode). The Cathode can be manufactuered from a variety of metal types, but the most common is stainless steel. The two electrode chambers are partitioned with a zirconium-aluminium ceramic membrane of low filtration capacity

to prevent admixture of the anodal and cathodal solutions (Figs 8 and 9), and has a capacity to withstand trans-membrane pressures of 1 atmosphere. This is the core for the ion-selective partitioning in the reactor cell. The resulting anodal and cathodal chambers are effectively isolated with elastic sealing rings and non-conducting dielectric fittings which house the inlet and outlet ports, and which in turn control the fluid flow dynamics through the separate chambers (Fig 10) (Bakhir *et al.*, 1999; Bakhir and Zadorozhny, 1999[a]; Marais and Brözel, 1999; Marais, 2000; Zinkevich *et al.,* 2000; Guentzel *et al*., 2008).

While a variety of prototypal variations have evolved since the first reports of the ECA process (Fig 7), the current commercial reactor used in the various studies of this report, represents the third generation of design and is referred to as the FEM 3 (Fig 8). In contrast to the classical membrane type cell (saturated brine and anodal feed), hypotonic saline is fed into the cathodal chamber of the FEM 3 reactor and exits from the same as catholyte (Figs 8 and 9). The porosity of the membrane permits the unimpeded flow of the saline from the cathodal into the anodal chamber, and anolyte will be generated and harvested concurrently. Dissociated cationic ions are partitioned within the cathodal chamber, and their conjugate anions will be selectively retained within the anodal chamber, this due primarily to the electrical charge gradient that is established between the two electrodes (Bakhir *et al*., 1999). Under the abovementioned production conditions, the two separately harvested electrochemically activated solutions display exaggerated physicochemical parameters i.e. either maximally acidic or alkaline pH, with markedly elevated oxidising or reducing REDOX values respectively. However, due to the specific hydrodynamic features of the different chambers, as well as the design of the solution reticulation system outside of the reactor, a portion of the catholyte effluent may be redirected as an additional influent stream into the anodal chamber. This incremental redirection of the catholyte stream into the anodal chamber, results in a progressive pH shift of the effluent anolyte stream from acidic towards neutral. Alkaline anolyte will be produced when the bulk of the catholyte stream is redirected into the anodal chamber. Thus, the customisation of the specific physico-chemical parameters of the anolyte solution is predominantly achieved by adjusting the proportion of the catholyte stream which is redirected into the anodal chamber.



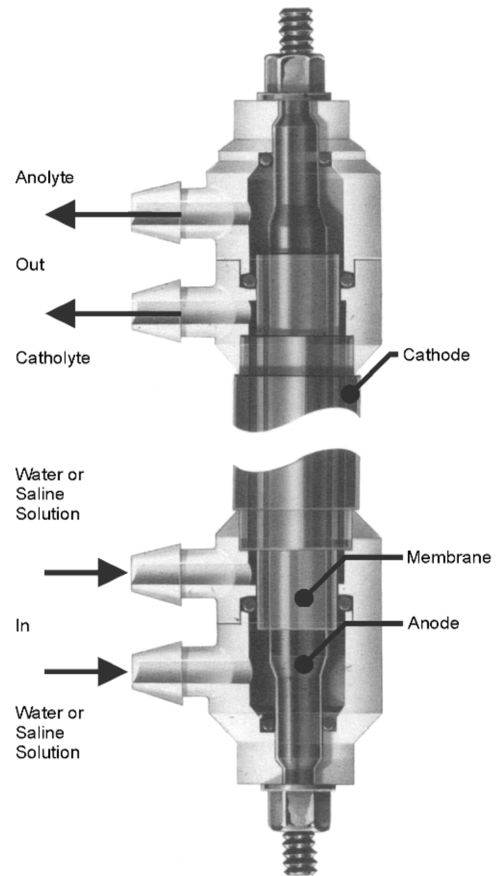


Figure 7. Design and components of FEM 2. Note External Anode (Bakhir, 1997)

Figure 8.Design and components of FEM 3. Note External Cathode (Bakhir, 1999)

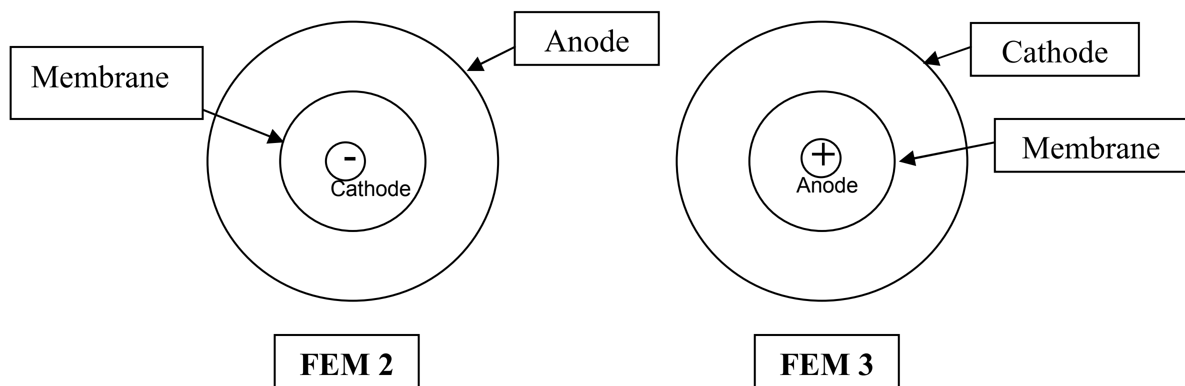


Figure 9. Cross sectional schematic of the different FEM designs describing the co-axial configuration of the electrodes and the differences between the two types of FEM reactor (Rowe, 2001).

Additional adjustments to the quality of the anolyte solution can be effected by changing amongst others, the intra-reactor chamber pressures, degree of mineralization of the feed solution, specific type of the catalytic salt reagent, influent saline feed temperatures and reactor flow rates (Hsu, 2005). While the changes in REDOX described an inverse relationship to the flow rate through the reactor, the magnitude of the REDOX shift was shown to be independent of the salt concentration of the feed solution. Conversely, the quantity of total residual chlorine formed during the reaction was directly related to the degree of salinity of the influent solution as well as the flow rate through the reactor (Hsu, 2005).

The range of electrolysis efficiency from 23-51% as measured by the reduction rate of chloride ions has been shown to be dependent on both the solution flow rate through the reactor and the salt concentration in the feed solution. Increases in both variables resulted in a substantial reduction in electrolysis efficiency (Hsu, 2003). Further manipulations to the process, such as the double activation of the anolyte solution can be achieved by feeding anolyte as opposed to saline or catholyte solution into the anodal chamber of the terminal reactor.

Thus a variety of hydraulic configurations are possible, all of which are designed to achieve specific objectives. The specific assembly and hydraulic configuration of the reactors within a generating system will dictate the specific type of solution that will be generated. Solutions can thus be tailored and optimised for distinctive or unique applications.

1.5.5.5 Physical and chemical activities of ECA solutions

Based on studies of ECA solutions generated using different types of electrolysis reactors, a number of theoretical postulates have been forwarded regarding the properties of ECA solutions.

Three distinct groups of features have been proposed to describe the distinctive physical and chemical characteristics of anolyte and catholyte solutions. These comprise:

1) Stable products of classical electrochemical reactions, i.e stable acids and bases,
2) Highly reactive unstable products including free radicals, with a half life of up to several hours, and
3) Longer-lasting quasi-stable structures which are formed in association with the electric field adjacent to the electrodes' surface, which either exist as free structural complexes or as hydrated membranes of ions, molecules, radicals and atoms (Prilutsky and Bakhir, 1997).

The determinants of the first group influence the pH value of the electrolyte and confer the acid and alkaline properties of the ECA solutions. Aqueous acids and their conjugate bases are the most versatile and universal catalysts of organic reactions and specific acid-base catalysis is defined as the rate enhancements which are proportional to the relative concentrations of H^+ and OH^- ions respectively (Lehninger, 1975).

The factors of the second group enhance the oxidant and reducing properties of the solutions. These comprise highly reactive and unstable products, including free radicals and other active ion species, having a typical lifetime of less than 48 hours. Included here would be electrically and chemically active micro-bubbles of electrolytic gases, 0.2 - 0.5 µm in size, at concentrations up to 10^7 ml $^{-1}$ distributed uniformly throughout the solution. This phenomenon accords with the reported response of pure water to a low density electromagnetic field, wherein an increase in the concentration of dissolved gases and an increased reactivity due to singlet oxygen ($^1O_2$) and free radical formation (eg. OH·) have been described (Chaplin, 2007). The presence of dissolved gases – hydrogen in the cathodal chamber and oxygen in the anodal chamber have also been independently confirmed by Shirahata *et al*. (1997), Hanaoka (2001), Hsu (2005) and Stan *et al*. (2005).

The factors of the third group are said to comprise quasi-stable structures. These are structures formed at or near the electrode surfaces as a consequence of the very high voltage drop (~ 10^6 volts/cm) at the electrode:electrolyte interface. This charge differential is proposed to result in the modification of the energetic interfaces between the interacting atomic and molecular components of the solution and will result in the formation of free standing structural complexes of hydrated membranes

or flat surface nanobubbles which will form around the dissolved ions and molecules (Chaplin, 2007). As a consequence of the previously proposed 'de-structurisation', the size of these water clusters will be reduced from 10-13 to approximately 5-6 molecules per cluster (Bakhir, 1999). This feature relates to a shift away from hydrogen bonding and has been confirmed with Molecular Dynamic Light Scattering studies (Chernikov *et al*., 1999). All of these features are proposed to contribute to the catalytic and biocatalytic properties of the water. Hanoaka (2001) has reported on the paradoxically protracted potentiation of superoxide dismutation activity when the reductant solution was combined with commercial antioxidants. Despite having no intrinsic Catalase or SOD-like activity, the catholyte still retained exaggerated antioxidant properties and this phenomenon is attributed to the extremely stable dissociation activity of water after electrolysis. The second and third group of factors are proposed to be unique to the production conditions of electrochemical synthesis. Additionally it is proposed that the replication of this effect would not be feasible under classical physical or chemical procedures (Bakhir, 1999).

1.5.5.6 Effect of Mineralisation on ECA solutions

The abovementioned product classes are proposed to be categorised as follows:

1 : stable chemical compounds, acids and bases,

2 : unstable / metastable highly active species,

3 : structural anomalies of water.

The relative contributions of mineralization to the different classes of activation products are described as in Table 1.

Table 1. Proportionate roles of the different reactive product categories arising from ECA solutions of varying mineralisation (Prilutsky and Bakhir, 1997).

Water Type	Mineralisation (g/l)	Relative Role
Ultra fresh	<0.3	$3 > 2 > 1$
Fresh	0.3 – 1.0	$3 \geq 2 > 1$
Hypotonic saline	1.0 - 3	$3 = 2 \geq 1$
Saline	3 - 10	$3 < 2 \leq 1$
Hypertonic saline	>10	$3 \leq 2 \leq 1$

From the above it is evident that in order to promote the generation of highly reactive unstable and metastable species, the electrical conductivity and hence the mineralisation (salt concentration) of the non-activated electrolyte solution must be kept below 3g/l. During the electrolysis of a strongly hypertonic saline solution, the substantial shifts in the pH and ORP values can be equated directly to that of conventional electrochemical syntheses wherein large quantities of concentrated acids or alkalis i.e. chlorine, sodium hypochlorite and sodium hydroxide respectively, are formed (Leonov, 1997; Bommaraju *et al.*, 2007).

When simplified, the main processes that may theoretically occur in an ECA reactor with a low mineralisation electrolyte can be expressed in the following way:

1) oxidation of water at the anode: $2H_2O - 4e^- \rightarrow 4H^+ + O_2$;
2) reduction of water at the cathode: $2H_2O + 2e^- \rightarrow H_2 + 2OH^-$;
3) formation of gaseous chlorine in chloride solutions at the anode: $2Cl^- - 2e^- \rightarrow Cl_2$;
4) formation of highly active oxidants in the anodic chamber: Cl_2O, ClO_2, ClO^-, $HClO$, $Cl\cdot$, $O_2\cdot$, O_3, HO_2, $OH\cdot$;
5) formation of highly active reductants in the cathodic chamber: OH^-, $H_3^-O_2^-$, H_2, $HO_2\cdot$, HO_2^-, O_2^- (Bakhir, 1999).

The creation of high concentrations of reactive oxidant species and free radicals in the anodal chamber results in a solution with marked biocidal properties. Simultaneously, the enhanced reduction in the cathodal chamber generates a saturated solution of reductants with properties of distinctive adsorption and detergent capacity (Bakhir, 1999, Hennion, 2006; Hsu, 2006).

While Suzuki *et al.* (2002) report on the presence of both hydrogen peroxide and hydroxyl radical in acidic EO water using Electron Spin Resonance (ESR) spectroscopy, more recent investigations into the generation of hydroxyl and superoxide radicals in electrolyzed water solutions failed to confirm the generation of either of the two reactive oxidant species using ESR. While the authors acknowledge that the chemistry between 5,5-Dimethyl-1-pyrroline-N-Oxide and the ECA solutions is unknown, their studies also failed to confirm the presence of chlorine dioxide in

freshly electroactivated brine solutions (Stan and Daeschel, 2005; Stan *et al*., 2005). It is important to note that it is unknown what reactor design was used in the generation of the solutions, and it has been confirmed that reactor design and operational parameters will substantially influence the quality and constituents of the electroactivated solution produced (Sampson and Muir, 2002).

When the solutions of anolyte and catholyte are generated from brines of different mineralization levels, there are substantial shifts in both pH and REDOX levels. When the pH value of the anolyte solution is acidified, the REDOX correspondingly shifts to an elevated positive (oxidant) value, and conversely when the catholyte pH is increased, the REDOX is progressively reduced to an increasingly negative (reductant) value. When the pH and REDOX of non-activated aqueous solutions of common chemical reagents and water are measured, the parameters are distributed in a range from pH=0–12.5 and REDOX from (–100) to 700 mV depending on the type of electrode couple employed.

By way of an example, the following extremes of pH and REDOX values can be generated with solutions of chemical reagents under laboratory conditions:

1. Concentrated sulfuric acid: pH=0.3; ORP = + 680 mV, CSE;
2. Saturated KOH solution: pH=12.3; ORP = –60, mV; CSE;
3. Saturated chloride of lime solution $Ca(ClO)_2$: pH=11.5; ORP=750 mV, CSE.

(CSE– Chloride: Silver electrode as the reference electrode) (Prilutsky and Bakhir, 1999).

It is recognized that when the concentrated reagent solution is progressively diluted with water, the pH of the solution will gradually approach the pH value of the diluent. This shift in pH value is associated with an equivalent regression in the Oxidation-Reduction potential, and this covariant relationship equates to approximately 60 mV for every unit of pH change. Thus when an acid is diluted with distilled water and the pH value of the solution increases by 1.0, then the expected reduction in REDOX will be 60 mV. Conversely, when an alkali is diluted with water, a 1 unit increase in pH

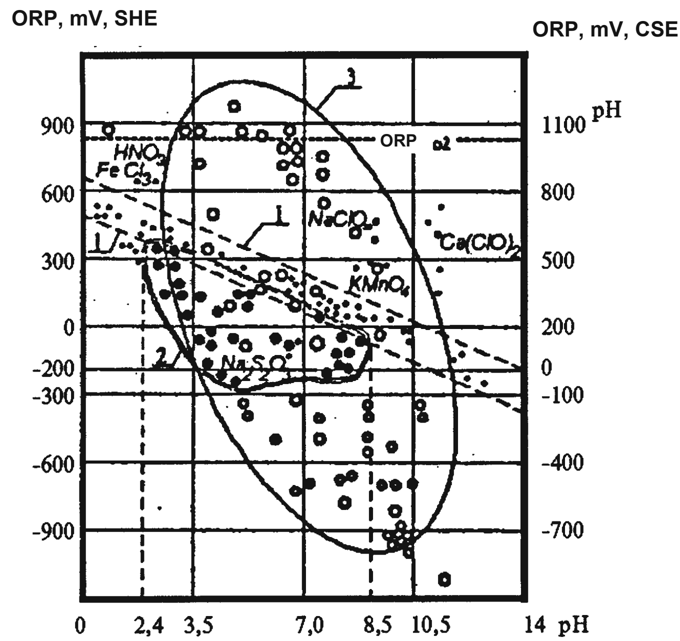
will correspond to an increase in the REDOX by 60 mV (Prilutsky and Bakhir, 1997; Bakhir, 1999). Thus there is a direct relationship between the changes in the REDOX of a solution for each unit change in the pH scale, and the plot of these covariant parameters on a coordinate system describes a linear relationship.

The distribution of pH and REDOX covariants of different compounds can be described according to the following categories:

- solutions of chemically pure inorganic compounds in distilled water without electrochemical treatment (marked as · (1))
- solutions of chemically pure organic substances in distilled water without electrochemical treatment (marked as • (2)), and
- ECA-solutions derived from a range of solutions from ultra-fresh water through to a concentrated saline solution, produced by unipolar electrochemical anodic or cathodic treatment (marked as °(3)) (Fig 12).

The parallel dotted line represents the correlation corridor for pH:REDOX covariants of inorganic compounds in solution. The area described by contour line 2 represents the conjugate pH:REDOX values for the solutions of organic compounds, and the area described by contour line 3 describes the distribution of pH:REDOX covariants measured in a variety of different ECA solutions (Prilutsky and Bakhir, 1997).

With progressive refinement of the design of the FEM, new generations of electrolytic cells have made it possible to consistently achieve unipolar (anodic or cathodic) activation of water with low levels of mineralisation (0.01-0.2g/l). Under these conditions, it has been shown that the production of high concentrations of acidic or basic electrolytic products do not occur (Prilutsky and Bakhir, 1997).



Legend: SHE – Standard Hydrogen electrode, CSE – Chloride Silver electrode.

Figure 10. Distribution of covariant pH and ORP (REDOX) values of inactivated organic and inorganic compounds in solution versus that of ECA solutions (Prilutsky and Bakhir, 1997).

Notwithstanding, the anodic and cathodic treatment of fresh, ultrafresh or even distilled water leads to the formation of anolyte or catholyte solutions that have heightened pH and exaggerated REDOX values relative to that of equivalent non-activated acidic and alkaline solutions respectively. This process is associated with the formation of covariant pH and REDOX combinations in the Anolyte and Catholyte solutions which cannot be replicated with conventional chemical reagents in the absence of exposure to electrochemical activation (Prilutsky and Bakhir, 1997).

1.5.5.7 Attributes of ECA solutions.

Common or potable water which is subjected to magnetism, sonic treatment, agitation, exposure to light, heating or cooling, and freezing with subsequent melting, attains new qualities which affect the kinetics of chemical reactions occurring within it, as well as its dissolution and washing properties.

In an ECA solution, the dissolved molecules undergo considerable structural transformation for an extended period of time, and through relaxation, there is a progressive reduction in its reactive capacity until it reaches an equilibrium state. Since it is not possible to determine the exact nature of the thermodynamic disequilibrium of solutions in an electroactivated state, the quantification and qualification of the ECA phenomenon relies largely upon an indirect description, where the difference in nett effect between non-activated and activated solutions is contrasted. The polar association of water molecules into clusters is predominantly due to the van der Waal's forces of attraction, and as this energy of association is largely negligible (8-20 kJ/mol), it cannot prevent the destruction of the aggregates of water molecules which occurs during electrochemical activation (Prilutsky and Bakhir, 1997).

Following the destruction of the water molecule aggregates, the aqueous medium becomes comprised of an increased proportion of water mono-molecules which have substantially heightened chemical reactivity. This feature manifests as a change in viscosity, diffusivity, heat conduction, electrical conductivity, catalytic activity, Oxidation-reduction balance, and surface tension (Bakhir, 1999).

1.5.5.8 Types of ECA Solutions

According to the inventor, the ECA solutions may be classified into four distinct various types:

i) A - acidic anolyte
pH: <5,0
REDOX: ≥ +1200 mV CSE
Active species: $Cl_2 > HClO > HCl > HO\cdot_2$
This solution is produced when there is no catholyte feedback or recirculation into the anodal chamber, and where there is a high degree of mineralisation (>5 g/l NaCl). Chlorine gas is evolved, the solution is highly oxidising. Both effluent products are mostly stable.

ii) AN - neutral anolyte

pH: 5,0 - 7,0

REDOX: +700 → +900 mV

Active species: $HClO$ > ClO_2, ClO^-, O_3 > $HO\cdot$, $HO\cdot_2$, HO^-_2, H_2O_2, $^1O_2$, $Cl\cdot$, $HClO_2$, $HO\cdot$, $O\cdot$.

In the generation of this solution, some catholyte has been recirculated into the anodal chamber and the mineralisation is generally low (<3 g/l NaCl). Under these production conditions, the formation of highly reactive and unstable radical species will predominate.

iii) ANK – neutral-alkaline anolyte

pH: 7,2 - 8,2

REDOX: +250 → +800 mV

Active species: ClO^- > $HClO$ > $HO\cdot_2$, $HO\cdot$, H_2O_2, $^1O_2$, Cl^-

Under these conditions the majority of the catholyte produced is recirculated into the anodal chamber, resulting in a higher pH. The solution retains its oxidising properties and has similar properties to AN, but it displays a shorter period of activation.

iv) K - electrically activated alkaline catholyte

pH: >9,0

REDOX: -700 → -820 mV

Active species: $NaOH$, O^-_2, $HO\cdot_2$, HO^-_2, OH^-, $OH\cdot$, HO_2^-, O^{2-}_2

This solution has a pH of 11-12 and is highly reducing. It is very active but the relaxation times are significantly shorter than that of the various Anolyte solutions ($t_{½} \leq 8$ hours).

v) KN - electrically activated neutral catholyte

pH: <9,0

REDOX: -300 → -500 mV

Active species: O^-_2, $HO\cdot_2$, HO^-_2, H_2O_2, $H\cdot$, $OH\cdot$

(Prilutsky and Bakhir, 1997).

As mentioned previously, neither hydroxyl radical, superoxide, ozone nor chlorine dioxide could be detected in the freshly generated oxidant solutions during targeted ESR studies (Stan and Daeschel, 2005; Stan *et al*., 2005). While Kimbrough et al., (2006) confirmed the presence of hydrogen peroxide in both the anodal and cathodal solutions by colourimetric titration with ammonium molybdate and sodium thiosulphate, the analysis of the alkaline antoxidant cathodal solution with ESR similarly failed to detail the presence of any ROS during the abovementioned investigation However while all of the studies readily confirmed the presence of both hypochlorous acid and hypochlorite molecules in a range of ECA solutions of differing pH values, until these and similar studies are standardised against a single reactor design type i.e. FEM, with directly comparable operation parameters, definitive conclusions as to the constituent composition of the electrolyzed oxidizing solutions relative to FEM derived solutions will remain questionable.

As with anolyte, the characteristics of catholyte can similarly be varied. Aside from chemical reagents, the catholyte solutions contain structural water clusters that are reduced in size to ≈ 5-6 water molecules and these have been shown to readily permeate through biological tissue. The charge on these clusters is proposed to be due to the presence of a surrounding negatively charged electron cloud (Prilutsky, 1997).

The co-axial arrangement of the electrodes has been engineered so as to create an electrical charge gradient or electric double layer (EDL) equivalent to a gradient of millions of volts/cm at both the anodal and cathodal electrode surfaces (Prilutsky and Bakhir, 1997). During the period of exaggerated relaxation activity, the ECA solutions can be applied as a direct substitute for conventional chemical reagents and regimens. In contrast to these chemical reagents, the final ECA products revert to the benign equivalent of the initial pre-activation solution i.e. dilute brine. The rate of relaxation decay or reversion to the pre-electroactivated state will vary dependent on the solution type and may be as short as a few hours (Catholyte) or it may extend to several months under optimal storage conditions (Anolyte).

Following the initial reports on the unique activities of the solutions generated by the early prototypal devices, considerable refinement has been made toward the optimisation of the dimensions and composition of the constituents of these

electrolytic cells in order to achieve enhanced productivity, reliability and sustained economic efficiency (Bakhir *et al*., 1999)

1.5.5.9 ECA DEVICES

Currently the on-site ECA electrochemical systems may, depending on their field of application, be categorised as follows:

1. Devices for generating liquid and gaseous chlorine - On-site Chlorine Generators e.g. AQUACHLOR-type.

2. Devices for generating electrochemically activated mixed oxidant solutions e.g STEL-type (Fig 9).

3. Devices for the treatment of drinking water e.g EMERALD-type.

Table 2. Classification of ECA devices (Bakhir, 1999).

Device type	Oxidant production capacity / hour	Salt usage per g of oxidant	Power consumption per hour
AQUACHLOR On-site chlorine generator	1000gm/hr	1.8 gm	3, 42 kw/hr
STEL On-site Mixed oxidant generator (Fig 11)	500gm/hr	1.0 gm	3,5 kw/hr
EMERALD* On-site water purifier	Nil *	Nil*	0.5-1 watt x hour/lit

* - The Emerald device uses electrolysis for the direct intra-reactor microbial elimination, removal of heavy metals and toxic organic compounds for the production of purified potable water. No brine is added and it produces an active oxidant concentration of 1-10 mg/l. The units are predominantly designed for house-hold use.

1.6 Conclusions

While water has systematically been regarded as a benign solvent or space-filler, its unique behaviour and distinctive physicochemical attributes, intrinsically facilitates its energetic manipulation. These features permit the creation of a diverse array of

previously unreported reagents with anomalous properties which exist exclusively in an electroactivated state.

The mechanisms of conventional brine electrolysis have been described in terms of predicable chemical pathways with quantifiable energetic exchanges. However the classical laws of thermodynamics have been shown to be substantially inadequate when it comes to describing the Electrochemcially activated state. To this end, the energy dynamics of the ECA electrolyte solutions can really only be described by the results that these anomalous energy states effect when compared and contrasted against equivalent applications with non-electrochemically activated solutions.

While an extensive array of electrolysis reactor systems have been manufactured, most designs have been geared to the commericial production of chemical compounds. Outside of these large industrial electrolysis systems, a variety of scaled-down variants have also been reported to electrolyse dilute brine solutions to produce solutions with elevated REDOX. However, most of these solutions do not subscribe to the full extent or range of anomalous attributes that have been ascribed to the coaxial aligned reactor capability.

Thus the sustained generation of the anomalous electroactivated state has required the design and fabrication of the refined FEM reactor configuration. Aside from reactor design, the ECA technology also requires operation under narrowly defined limits of input quality and quantity. It is only through consistent adherence to these prescribed standard operating instructions that ECA solutions of reliably repeatable quality and performance capabilities can be produced.

Given that the original literature is highly theoretical and oftentimes speculative, the claims relating to the range of reactive radical species that are produced during the electroactivation process have been found to be wanting and recent investigations with sophisticated analyses have not been able to substantiate the original claims.

Notwithstanding, the elevated Oxidation-Reduction potential generated during the electroactivation process has been substantially corroborated by independent studies, and while the unique differentiating attributes of the ECA solutions relative to those of earlier electrolysis devices remains largely untested, a growing body of evidence would suggest that the coaxial reactor design has the capability to selectively manipulate the properties of water in dilute brine solutions.

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Chapter 2

Microbial energetics

2.1 Introduction

Pursuant to the concepts developed on the physical and chemical behaviour of molecular water, this theme will be expanded to encompass the specific requirements necessary for the creation and maintenance of viability of all organisms and prokaryotic bacteria in particular.

All biological phenomena depend on the specific association of atoms and molecules. It is the precise ordered arrangement of these atoms and molecules which confers functional specificity and which by consequence, sustains viability. Aside from these fundamental atomic interactions, the behaviour of more complex molecules and compounds within a biological system has particular relevance to the nature of the structures that form and the physicochemical parameters that dictate their existence.

Regardless of the complexity of a bacterium, the cell is regarded as the true and complete unit of life. Living cells are composed of protoplasm which consists of a colloidal organic complex including proteins, lipids and nucleic acids which are enclosed in a limiting membrane or cell wall. Aside from the ability to reproduce and mobilise nutrients for energy metabolism, all living organisms have the capacity to respond to changes in their environment – a feature termed irritability. In contrast to all other life forms, the intrinsic versatility of bacteria to adapt to variable environmental conditions translates into the broadest range of physiological and biochemical potentialities yet described for any class of organism. This is reflected by their short-term ability to manipulate and control metabolic activity, regulate growth and in some instances to amend the details of their genetic material without compromising the viability and integrity of the organism (Pelczar and Reid, 1972).

In order to describe the intricate complexity of the physiological and biochemical processes that confer this adaptive flexibility, it is necessary to revert to an understanding of the basic building blocks that constitute the bacterium.

Given that the fundamental tenet for life remains the capacity to generate and utilise energy on a sustainable basis, the discussion on the specific molecular structure and functionality of the microorganism will be approached from an energetic perspective as opposed to a restatement of the long established biochemical descriptions of the protoplasmic building blocks.

All the molecular compounds that comprise the organic colloidal matrix of the bacterial cytoplasm arise from the forces of attraction that bond the various atomic elements into defined functional molecular structures. All bonds are energy based and the more energy involved in the reaction, the stronger the bond that is formed. These reactions are characterised by a change in the free energy of the system (ΔG).

$$\Delta G^{0'} = -nF\Delta E_0'$$

where $G^{0'}$= Standard free energy, F = Faraday's constant (96,500 coulombs), n = number of electrons transported, E_0' = standard electron potential (Caldwell, 1995).

The standard free energy of reactions is best understood in terms of basic thermodynamic principles. The first law of thermodynamics states that energy is conserved i.e. neither created nor destroyed. Any change in the energy of a system (ΔE) requires an equal and opposite change in its surroundings, and equates to the difference between the heat absorbed (q) and the work done by the system (w) (VanDemark and Batzing, 1987; Zubay *et al.*, 1995).

$$\Delta E = q - w$$

This equation gives rise to the concept of enthalpy and describes the relationship between the change in enthalpy (ΔH), energy, pressure (P) and volume (V) where,

$$\Delta H = \Delta E + \Delta(PV)$$

The second law of thermodynamics states that the universe moves from states that are more ordered to states that are more disordered. This thermodynamic function is termed Entropy and the change is denoted as ΔS (Zubay *et al.*, 1995).

It also states that only part of the energy released in a chemical reaction is available for the performance of work. The total energy or Enthalpy (H) released during a chemical reaction is composed of the energy available for work i.e. free energy (G) plus that which is not available for work and which is termed Entropy (S) (VanDemark and Batzing, 1987; Kotz and Purcell, 1991).

Enthalpy is conventionally described in electronic terms and entropy in terms of the translational and rotational energies of molecular alignment, and the relationship between the two entities has been shown to be dependent on the physical environment where T is the absolute temperature in degrees kelvin.

$$\Delta S = {}^{\Delta H}/_{T}$$

In water solutions, solvation refers to the interaction of a solute with a solvent, and the reduction of entropy associated with solute dissociation is due to the formation of hydrogen bonding or an increase in the clustering of water molecules due to induced changes in the polarity of the solute. Since reactions do not occur in isolation from the surrounding environment, the Gibbs equation has been used to describe the composite relationship between the three concepts.

$$\Delta G = \Delta H - T\Delta S$$

(Kotz and Purcell, 1991; Zubay *et al.*, 1995).

The Free Energy changes involved in the different types of chemical bonds are quantified as follows:

Covalent bonds: 104 kcal/mol,

Hydrogen bonds: 2-10 kcal/mol,

Van der Waals forces: 1-2 kcal/mol.

(Lehninger, 1975; Stumm and Morgan, 1996)

For comparative purposes, the standard free energy of formation of water (ΔG^{o}_{f}) or the energy required to form one mole of water molecules is -56.69kcal/mol. The negative ΔG^{o}_{f} is an indication of the spontaneous nature of the reaction and describes an exergonic reaction (Nester *et al.*, 1973).

While covalent bonds are the most robust and stable forces of attraction between molecules, hydrogen bonding occurs when a positively charged hydrogen atom involved in a polar covalent bond, interacts with the negative portion of another covalently bonded atom. All atoms will interact with each other irrespective of their chemical nature, charge properties or their involvement in other chemical bonds. Thus any and all atoms are attracted to a defined and characteristic distance between themselves due to the intrinsic nature of the charges of the individual atoms. While these van der Waals forces of attraction are relatively weak, the interactions of the bonds are additive and with incremental levels of attraction, will result in robust, definitive and complementary structures with distinctive specificity of molecular alignment that ultimately dictates their functional biological significance.

2.2 Molecular structures

Proteins arise from the assimilation of amino acids in a polymeric sequence. This peptide bonded sequence of amino acids is referred to as the primary structure. As a result of folding of the chain, a three dimensional helical structure arises wherein the development of hydrogen bonding between the side chains confers progressive stability and functional specificity. This structural stability is further supplemented by the development of van der Waals forces of attraction, disulphide covalent bonds and hydrophobic bonds, the later of which serves to isolate the non-polar hydrocarbon side chains within the overall structure, thereby limiting their exposure in an aqueous environment. These weak bonds which are responsible for the stability of the three dimensional structures are readily disrupted by the introduction of energy (Liao *et al.*, 2007). Conventionally, this has been associated with heat energy, but non-thermal energy sources such as radiation have been shown to exert similarly disruptive effects. When the protein loses the three dimensional structure, here is a consequential loss of functional integrity (Kotz and Purcell, 1991).

DNA exists as double stranded helix of nucleotides where the stability of the structure is conferred by a large number of relatively weak hydrogen bonds. As with proteins, the introduction of sufficient energy, (eg. a temperature increase to 80°C) is capable of disrupting the double stranded structure and impairing its functionality.

Lipids are the product of glycerol bonding to hydrocarbon chains of variable length. Their relative insolubility in water is due to the preponderance of non-polar groups. Where lipids bond with protein linkages to form lipoproteins, the resultant molecule relies primarily on van der Waals forces of attraction and not covalent bonds to remain functionally structured. In converse, the structure of membrane associated lipopolysaccharide molecules is predominantly maintained through covalent bonding (Kotz and Purcell, 1991). The lipopolysaccharide bilayer that forms from the specific alignment of the non-polar 'tails' and polar 'heads' when exposed to an aqueous environment, mirrors the structured arrangement which lipids display in biological unit-membranes. The different polarities of the lipid compounds in the cytoplasmic membrane that encapsulate the protoplasm, facilitates the unique biological functions of the different membrane fractions (Nester *et al.*, 1973).

2.3 Bacterial structures

The cytoplasm of bacteria is comprised of a highly concentrated solution of inorganic salts, sugars, amino acids and various proteins, and it is the ability of the organism to concentrate these molecules within an encapsulating barrier structure that permits the cell to maintain a constant intracellular environment under varying environmental conditions.

Conventionally there is a tendency for the concentration of these low molecular weight molecules to equalise across the membrane. The physicochemical forces which govern the bulk movement of water across a membrane, directly affect the shape and form of living organisms. These forces are detailed in the Gibbs-Donnan equilibrium, where the cell membrane is described as being freely permeable to ions and water but selectively impermeable to charged macromolecules (Hempling, 1981). The selective permeability of the cytoplasmic membrane prevents the free movement of these macromolecules out of the cytoplasm, and the consequent asymmetry in osmolarity will theoretically result in a net influx of water into the cell (Nester *et al.*, 1973). To counter this influx and to limit the distension of the phase boundary, the hydrostatic or turgor pressure will increase and this confers the distinctive cellular rigidity to the membrane bound bacterium (Hempling, 1981). This restriction to the selective flow of water gives rise to an osmotic pressure which in gram positive

bacteria with a rigid cell wall, may reach 25 atm (2.5MPa). The same osmotic pressure in gram negative bacteria encapsulated by a flexible membrane, only reaches 5 atm (500kPa) (Nester *et al*, 1973; Labischinski and Maidof,1994).

Movement across the membrane occurs either by passive diffusion or active transport. Passive movement is not energy dependent while active transport requires energy expenditure and results in an increased concentration of the selectively transported molecules within the cell structure (Nester *et al.*, 1973). The proteins of the permease transport system reside in the phospholipid bilayer of the cytoplasmic membrane and they select the ions that enter and leave the cell as well as predict their rate of transport. These hydrophyllic protein channels that traverse the lipoprotein bilayer allow the transport of hydrophyllic substances through an essentially hydrophobic bilayer (Caldwell, 1995).

These enzymes are energy transducers and convert the energy of metabolism into osmotic work. By this means selected ions are transferred across the membrane against their electrochemical gradients, and give rise to a change in the osmotic activity of the cytoplasm (Hempling, 1981). Thus any change to the energy status of the microenvironment of the cell, its membrane and the embedded protein mediated transport system, will have significant implications on the ability of the microbe to maintain its structural and functional integrity.

In bacteria, the cell wall or membrane determines the shape of the organism. In gram positive organisms with definite cell walls, the wall is comprised of a peptidoglycan (Murein) mucocomplex consisting of two subunits, N-acetyl muramic acid and N-acetyl glucosamine (Shockman and Höltje, 1994). The structure and strength of the cell wall is conferred by the covalent bonding of a chain of d-amino acids to the muramic acid which forms a rigid interconnected macromolecular structure (Nester *et al*, 1973). The rigidity of peptidoglycans is attributable to the restricted flexibility of the sugar chains and this is due to the limited rotation about the β 1-4 linkages, which precludes the abrupt bending, and hence distention of the chains (Labischinski and Maidof, 1994). Additionally the presence of teichoic acids add further support and structure through covalent attachments and these constituents are proposed to play a role in ion accumulation (Caldwell, 1995).

Conversely, bacteria with a cell membrane have an outer lipopolysaccharide layer which confers rigidity and shape to the envelope and protects the cell against osmotic lysis. The peptides in the peptidoglycan matrix of the membranes of gram negative bacteria are highly flexible and can be extended by up to 400% of their length during stress. They comprise of peptide dimers which display a limited degree of cross linking i.e. 25-30% as opposed to that of gram positive bacteria i.e. 70-90%. Thus the conditions experienced by bacteria during normal growth also correspond to a capacity of the bacteria to respond to adverse changes in osmolarity with a corresponding change in volume adaptation (Labischinski and Maidof, 1994).

The inner lipoprotein layer of gram negative bacteria contains trimeric aggregates of hydrophyllic proteins termed porins which facilitate the passage of both hydrophilic and hydrophobic molecules through the largely impermeable lipopolysaccharide barrier (Nester *et al.*, 1973; Hancock *et al*., 1994; Caldwell, 1995). Porins exercise a significant influence over the maintenance of the electro-osmotic gradient and the matrix porin consists of highly specific proteins with distinctive differences. Both OmpF and Phospho-Porin (Pho–E) porins permit passive diffusion of hydrophilic solutes up to a mass of 600 Da across the outer membrane, and both are highly stable and resistant to proteases and detergents. However the OmpF fraction has a large pore size and is weakly cation specific, while the Pho-E porin fraction has been shown to be strongly anion selective (Cowan and Schirmer, 1994).

Of importance to the sustained activity of porins, is the impact of energy fluctuations in the immediate microbial milieu, and artificial simulations using synthetic bilayers have detailed a voltage driven 'gating' phenomenon, wherein the OmpF porin has a pore closure potential of ~90mV while that of PhoE was reported to be ~100mV (Cowan and Schirmer, 1994). In both gram positive and negative cells, the inner cytoplasmic membrane acts as the real diffusion barrier and comprises the structures necessary for the respiratory chain, facilitated transport systems as well as the mechanisms for protein export (Benz, 1988). This innermost structure of the cell wall or membrane is unique to the prokaryotes, and is a critical structure for the maintenance of life. Aside from acting as the real diffusion barrier responsible for selective permeability, it also plays a role in cell division, sporulation, electron transport, ATP formation and DNA replication (Caldwell, 1995). More importantly, is

the fact that the intact membrane has been shown to be fundamental for optimal cellular energy transduction (Datta, 1987).

2.4 Energy conservation

The cytoplasmic membrane is recognised to be a cornerstone for the maintenance of normal cellular activity, and its ability to generate and mobilise metabolic energy as work for the maintenance of the electro-osmotic gradient remains pivotal to cellular integrity and viability. It is the universal function of all living organisms to have the capacity to conserve and use energy. As energy confers the ability to grow and replicate, sustained cellular viability can thus be restated in terms of this conserved energy affording the cell with the capacity to perform work (Robertson, 1983; VanDemark and Batzing, 1987).

All living forms derive energy from one of two ways, namely substrate level phosphorylation (SLP) and electron transport. SLP occurs in the cytoplasm and requires distinctive enzyme systems and produces a single high energy bonded molecule per unit of substrate degraded. Electron transport phosphorylation is a membrane associated activity and uses a common series of carrier molecules and associated enzymes to produce more than one high energy molecule per unit of electrons that is processed (Caldwell, 1995). Electron transport is an obligatory membrane associated process. If the components of the oxidative phosphorylation reactions were free in solution, the net result would be an exergonic reaction with heat generation (negative change in ΔG) and not the creation of a proton gradient which is capable of further work (VanDemark and Batzing, 1987; Caldwell, 1995).

In aerobic respiration, electron transport phosphorylation refers to a sequence of reactions in which an inorganic compound (electron-sink compound) is the final electron and hydrogen acceptor. In chemotrophic electron transport, oxidation of the last electron carrier is used to reduce a terminal electron acceptor. Thus there is a net loss of electrons from the system and a reduced compound is produced. In aerobes this reduced compound is water and derives from the reduction of half a molecule of oxygen with two protons (Hydrogen – H^+) and two electrons derived from the transport process.

Since anaerobes lack the majority of the coenzymes present in the cytochrome chain, when exposed to air they will convert hydrogen and oxygen to hydrogen peroxide instead of water with its consequential sequelae. Thus in contrast to aerobes, anaerobes utilise organic compounds as the terminal electron acceptor, and fumarate performs the equivalent function of oxygen and produces two reduced substances, namely succinate and propionate (Caldwell, 1995). In addition, anaerobes can also use nitrate (NO_3^-), sulphate (SO_4^{2-}) and CO_2 as electron-sink compounds (Nester *et al.*, 1973).

For each pair of electrons that passes through the membrane associated electron transport chain, one oxygen atom is reduced and four protons pass to the outside – two of these protons originally derived from substrate metabolism and two from water. This selective efflux of protons results in the cytosol of the cell becoming both electronegative and alkaline relative to the outside, and this active proton gradient is used to drive several additional energy requiring processes (Robertson, 1983; Caldwell, 1995; Zubay *et al.*, 1995). The generation of the Proton Motive Force (*pmf*) across the membrane is dependent on the selective extrusion of hydrogen ions to the exterior of the cell, and is a direct function of the difference in the hydrogen ion concentrations between the cell exterior and the cytoplasm (VanDemark and Batzing, 1987; Caldwell, 1995).

Thus the proton gradient is the major component of the *pmf* which is required for oxidative phosphorylation and ATP formation, and the stoichiometry of the phosphorylation products is dictated by the proton movements. The free energy exchange required for the synthesis of ATP depends on the ratio between the proton concentrations across the membrane, as well as the difference in electronic potential across the same (Caldwell, 1995; Zubay *et al.*, 1995). Thus the complex sequence of energy mobilisation steps required for the conversion of the products of substrate metabolism to that of high energy reserves necessary for further work capacity has been shown to be intimately reliant upon both the physical and electronic membrane integrity of the cell (Robertson, 1983; Caldwell, 1995; Zubay *et al.*, 1995).

The capacity to sustain the optimal energetic state of the cell is critically dependent upon its ability to maintain the requisite charge differential across the wall and/or

membranes, and as stated earlier, any substantive change to the electrical charge of the microenvironment of the bacterium will result in a critical disruption to this energetic homeostasis with potentially lethal consequences. All bacteria, irrespective of their surrounding environment, will attempt to maintain their intracellular environment at a neutral pH. However a range of adaptive variations have developed and it has been shown that acidophiles require a constant proton extrusion, while in contrast, alkalophiles require a continual proton influx in order to maintain intracellular neutrality. Consequently it has been found that that alkalophiles generate a *pmf* through the development of a membrane potential as opposed to a proton gradient, and conversely it has been shown that acidophiles do not possess the mechanisms to generate a proton gradient (Caldwell, 1995).

2.5 Response to environmental change

Bacteria survive in a heterogenous array of environments which are characterised by multiple physical and chemical determinants, and it is a constant requirement to remain optimally adjusted to shifts in these diverse environmental factors. It is logical that it is the combined interactions of the manifold environmental factors as opposed to the impact of any single factor which will determine the physiological range under which a microorganism will display the most optimal vitality and thus sustained viability. Ranges in the osmolality of the bacterial environment have revealed that bacteria with rigid cell walls are capable of displaying a far greater degree of tolerance to variations in osmotic pressure when compared to organisms encapsulated by a flexible cell membrane. Extreme anomalies exist wherein true halophiles specifically require high salt concentrations for maintenance of their structural integrity, and it is obligatory to use salt as a component of the growth medium to selectively culture the halotolerant *Staphylococcus aureus* organism (Caldwell, 1995; VanDemark and Batzing, 1987).

As a corollary, the direct availability of water or water activity (a_w) plays a significant role in the microbial requirements for growth. An example of this is seen where fungi can grow at an a_w of 0.8 while bacteria require a minimum a_w of 0.9. This results in grains being more susceptible to spoilage by fungi and mould than by bacteria (VanDemark and Batzing, 1987).

Shifts across a wide range of temperatures have been reported to select for distinctive population types with narrow temperature preferences i.e. thermophiles (>50°C), mesophiles (25-45°C) and psychrophiles (<20°C) (Nester *et al.*, 1973). Aside from the direct physical impact of temperature on bacteria, it also influences oxygen availability, pressure, pH, and moisture levels (VanDemark and Batzing, 1987). Notwithstanding the influence of these micro-environmental determinants, the more extreme limits of bacterial growth and viability have been shown to be fundamentally dictated by the acidity (i.e. pH) of the immediate microenvironment (Nester *et al.,* 1973; Caldwell, 1995).

While most bacteria have been shown to prefer a neutral pH, tolerances under extreme acidity (eg. *Thiobacillus* spp.- pH~2) and alkalinity (*Bacillus alkalophilus* pH~10.5) have been reported (Caldwell,1995). In acidic environments, the cell membrane blocks H^+ from entering and continually expels H^+ ions from the cell. Conversely, in alkalophilic and alkaline-tolerant micro-organisms, Na^+ ions are selectively excluded from the cells in order to maintain a near neutral internal pH. In addition, it has been reported that at lower pH values, there is an increased sensitivity to higher temperature (VanDemark and Batzing, 1987; Caldwell, 1995). When Alkalophilic organisms of the genus *Bacillus* are challenged with a shift in pH from 7 to 10, they responded by increasing the amount of polyanionic teichoic acids in their cell wall structure. This selective response was shown to result in an increase in proton retention near the membrane surface (Pooley and Karamata, 1994).

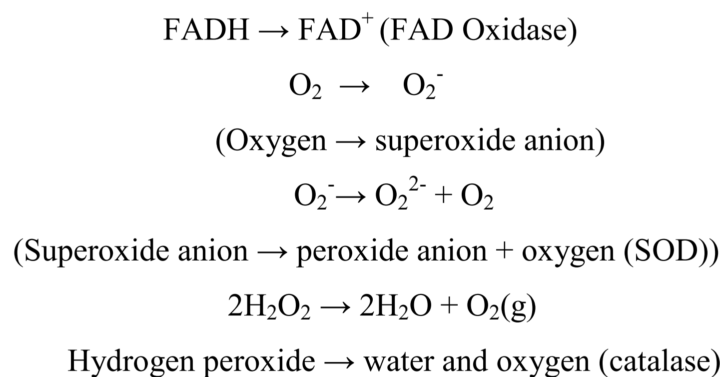
There are also distinctive pH driven effects on protein form and function, and Michaelis proposed that all proteins should be viewed as diprotic acids, where an acidic shift in pH results in the progressive ionisation of the protein with the evolution of a proton (Caldwell, 1995).Given the critical balance between the integrity of the low energy bonds that confer the tertiary structure of proteins and their functional specificity, any overt energy based intervention that would be disruptive to tertiary protein structure and thus physiological function, would have substantially adverse implications upon optimal metabolic activity and by consequence, microbial viability (Caldwell, 1995).

Aside from the specific hydrogen ion concentration which will govern the pH status, the energy quotient of a given thermodynamic system will also be described by the equilibrium state of the catalysed reactions, and this in turn will dictate the functional behaviour of the proteins. Thus for protein activities such as enzyme transport, the shape of the activity curve as a function of pH will approximate a bell curve with progressively more suboptimal activity on either side of the midpoint. Thus the optimal activity of a protein will correspond to a singly ionised condition, and it has been shown that the structural changes that affect the activity of proteins will predominantly correspond to changes in those molecular structures which contain oxidant-sensitive sulfhydryl groups (Caldwell, 1995).Therefore in a benign aqueous environment, the limits of the protein function will be primarily described by conventional chemical and physical determinants. Conversely the presence of increased concentrations of oxy- and hydroperoxi- radical species generated during and after exposure to oxidant ECA solutions will result in further adverse shifts in the reactivity of proteins and other equivalently complex macromolecules under fixed concentrations of H^+ and thus pH values. The effect of changes in pH on specific components within the cell membrane has long been established, and an increase in the pH in the vicinity of the membrane leads to disruption of the structure of the poly-anionic lipoteichoic acids. These are responsible for the sequestration of protons required for the development of the trans-membrane pH gradients, and if extreme, are suggested to block further growth potential (Pooley and Karamata, 1994).

The environment in which one isolates a given bacterium depends to a large extent on its nutritional requirements (VanDemark and Batzing, 1987). Aside from variations in the immediate elemental milieu which would determine the profile of nutrients available to the bacterium, variations in the concentration of oxygen is a significant determinant of the metabolic profile and hence the characteristics of the bacterial type encountered. Oxygen solubility increases at lower temperatures and hence obligate aerobes are best adapted to a psychrophilic growth regimen that reflects enhanced oxygen availability (VanDemark and Batzing, 1987). As with temperature and pH, there are distinctive ranges in oxygen concentration under which microbes are able to maintain metabolic activity. Bacteria can similarly be categorised in accordance with their ability to utilise different concentrations of oxygen and their characterisation will describe a distinctive range from obligate aerobes, microaerophiles, facultative

anaerobes, aerotolerant anaerobes through to obligate anaerobes (VanDemark and Batzing, 1987; Caldwell, 1995).

Due to the contribution of oxygen to the metabolic pathways and the likely evolution of toxic metabolites, the tolerance to the exposure of oxygen is dependent on two enzymes – Superoxide Dismutase (SOD) and Catalase. The substantive roles played by these two enzymes during aerobic metabolism, is confirmed wherein the oxidation of the flavoprotein molecule causes the release of oxygen which is converted to progressively more cytotoxic compounds including superoxide and peroxide anions:



$$FADH \rightarrow FAD^{+} \text{ (FAD Oxidase)}$$

$$O_2 \rightarrow O_2^{-}$$

(Oxygen → superoxide anion)

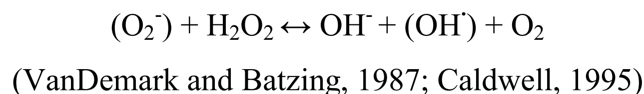
$$O_2^{-} \rightarrow O_2^{2-} + O_2$$

(Superoxide anion → peroxide anion + oxygen (SOD))

$$2H_2O_2 \rightarrow 2H_2O + O_2(g)$$

Hydrogen peroxide → water and oxygen (catalase)

Additionally, the reduction of oxygen may also involve the addition of only one electron as opposed to two, thus resulting in the formation of the toxic superoxide radical (O_2^{-}), which may react with peroxide (H_2O_2) to produce the even more toxic hydroxyl radical ($OH^{\cdot}$):



$$(O_2^{-}) + H_2O_2 \leftrightarrow OH^{-} + (OH^{\cdot}) + O_2$$

(VanDemark and Batzing, 1987; Caldwell, 1995)

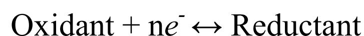
The formation of the superoxide and peroxide anions are an obligatory consequence of oxybiontic metabolism and the ranges of tolerance to oxygen concentration relate to the relative presence of the SOD and catalase enzymes. Obligate aerobes contain both SOD and the haem-type catalase enzymes, while aerotolerant anaerobes only contain SOD (VanDemark and Batzing, 1987; Caldwell, 1995). Almost as a default definition, aerobes exist primarily as a consequence of their enzymatic capacity to protect their metabolic processes and cellular structures from the toxic effects of oxygen and its metabolites.

The significance of this metabolic enzyme profile to the survival of the bacterium is that it contributes to the prediction of the likely capacity of specific bacterial categories to tolerate different types of biocidal intervention, some of which may induce oxidative stress. Thus exposure of an anaerobic bacterial population to an oxidising agent such as hydrogen peroxide (H_2O_2) would have a considerably more detrimental effect than if an aerobic or a facultative anaerobic population were to be exposed to the same (Caldwell, 1995).

2.6 Oxidation- Reduction Potential (ORP)

Oxidation-Reduction Potential (ORP) or REDOX is referred to as the measure of electronic pressure in a system, and is described as the behaviour and movement of electrons in a given medium (Thompson, 1995). ORP correlates to the postulate of 'electrochemical potential', and denotes 'the level of free energy relative to the number of moles of a given substance in the system'. By definition the 'electrochemical potential' is equivalent to the amount of free energy of a biochemical reaction required for the transfer of electrons from donor compounds to acceptor compounds. Redox potentials are thus thermodynamic properties that depend on the difference in free energy between the oxidised and reduced forms of a molecule (Zubay *et al.*, 1995).

For a redox reaction to occur there needs to be a molecular 'couple' where an electron-acceptor gains an electron and as a consequence, becomes reduced, i.e.



$$\text{Oxidant} + ne^- \leftrightarrow \text{Reductant}$$

where ne^- is the number of electrons transferred in the reaction
(Lehninger, 1975; VanDemark and Batzing, 1987).

Thus the tendency of a reducing agent to loose electrons or an oxidant to accept electrons describes the Oxidation-Reduction Potential of the system and is directly equivalent to the electromotive force (emf) (Lehninger, 1975). The removal of either a hydrogen ion or an electron from a given compound results in the compound becoming oxidised and it would thus have undergone an oxidation reaction. A decrease in free energy is primarily associated with oxidation reactions. These

electrons do not remain free but combine immediately with another compound which accepts the electrons thus becoming reduced (Prilutsky and Bakhir, 1997). Compounds with high hydrogen content are generally highly reduced, while compounds with low hydrogen content i.e CO_2 are highly oxidised. It thus obvious that reduced compounds contain more energy than oxidised compounds (Nester, *et al.*, 1973).

The ORP can be calculated using the Gibbs' formula ($\Delta G = \Delta H - T\Delta S$), and is measured in millivolts and is denoted as φs. (Prilutsky and Bakhir, 1997). Thus the ORP is a measure of electronic pressure (either positive or negative) produced by a liquid medium relative to the material of the measuring electrode and the reference system (VanDemark and Batzing, 1987; Zubay *et al.*, 1995; Prilutsky and Bakhir, 1997). The amount of energy released during a particular oxidation step is calculated from the difference in standard oxidation potential between the system that is oxidised and the system that it oxidises (Caldwell, 1995). The Nernst equation expresses the relationship between the REDOX potential of a standard REDOX couple, its observed potential and the concentration ratio between its electron donor and electron acceptor species.

$$E_h = E'_o + 2.303RT/nF \log {}^{[\text{electron acceptor}]}/_{[\text{electron donor}]}$$

where E_h = the observed redox potential, E'_o = the standard redox potential (ph 7), T = 25°C, (1 M), R = Gas constant (8.31), T = Temperature, n = number of electrons being transferred and *F* = Faraday constant (23.062 cal) (Lehninger, 1975; Zubay *et al.*, 1995).

Thus in aqueous electrolyte solutions which contain the core components of the [Ox]:[Red] couples, the electronic pressure is generated by the admixture of the oxidized and reduced components of the individual redox-pairs within the water medium, as well as the presence of evolved gases. The electronic pressure is thus determined by the activity and concentration of the free electrons in the solution as well as the cumulative transport energy of these free electrons (Prilutsky and Bakhir, 1997).

Negative values are associated with strong reductants and conversely a positive redox potential details that of strong oxidants. Redox reactions will follow specific sequences that are governed by the relative strengths of the standard redox potential of the redox couple. Spontaneous electron transfer requires conditions to be thermodynamically favourable, but also requires that the carriers should be able to make direct contact. Thus, in general, electrons will flow spontaneously in the direction of the more positive potential (Zubay *et al.*, 1995). Free electrons are present in any medium irrespective of whether exothermic or endothermic reactions take place in it. Any electrolytic dissociation of a solute in water is accompanied by an electron transfer. These electrons are reported to exist in solution as an ‘electron cloud’ or as a ‘rarefied electron gas’, and depending on the polarity of the in-contact electrode, it will assume the role of either electron donor or acceptor relative to the movement of the free electrons (Prilutsky and Bakhir, 1997).

The activity of the dissociated ions in the solution will induce structural changes to the composition of the dissolving water molecules, and the altered electronic state will thus translate as a multifactorial modification of the solvent. In accordance with generally accepted concepts, these structural modifications to the water molecules and the associated shifts in their energy state, will revert or ‘relax’ to their initial states when the reagent additive or extrinsic energy source which caused the modification, is removed. In addition, there is speculation that the solvent water retains or preserves a ‘memory’ of its original energy state, and the fundamental principles of homeopathy are founded on this premise (Prilutsky and Bakhir, 1997).

The REDOX potential of a sample of the reactive species generated during brine or saline electrolysis is described below (Rowe, 2001):

ClO_2 1500 mV at pH<5
Cl_2 1400 mV at pH<7
O_2 1200 mV
HOCl 1500 mV at pH<5
HOCl 850 mV at pH 7
OCl^- 640 mV
OH^- 400 mV

The decisive role of pH in determining the magnitude of the standard free energy of different molecules is confirmed in the above schedule.

Water has the unique capability to act as both a hydrogen donor and acceptor, and its capacity to form multiple hydrogen bonded structures stands in contrast to other polar molecules which are only capable of forming a single hydrogen bond (Duncan-Hewitt, 1990). Oxidation-Reduction-Potential is thus a cumulative parameter, and it is dependent on all of the components of the aqueous phase. ORP thus integrates all of the oxidative and reducing species in the solution. Oxygen has a strong tendency to accept electrons and to become reduced. It is thus a potent oxidising agent and has a strongly positive ORP (VanDemark and Batzing, 1987).

It has been reported that different bacterial species display varying susceptibilities to changes in oxidation-reduction potential and that each species exists within a specific ORP range where adaptive growth is possible (Lotts, 1994; Prilutsky and Bakhir, 1997; Kimbrough *et al*., 2006). An environment with a highly positive ORP is essential for the optimal growth of obligate aerobes, however it is possible to culture aerobic bacteria in the absence of oxygen provided other strong electron acceptors i.e. nitrates and sulphates, are present. Conversely a reducing environment with a negative ORP is required for the sustained growth of obligate anaerobes. Thus ORP will limit the environmental range which can sustain the growth of microbes, and the correspondence of the same with variable respiratory characteristics is detailed in Table 1.

Table 1. ORP ranges and growth limits for different microbial respiratory types (VanDemark and Batzing, 1987, Venkitanarayanan *et al.*, 1999).

Respiratory type	ORP limits (mV)	ORP range (mV)
Obligate aerobes	+200 ↔ +750	550
Obligate anaerobes	-100 ↔ -700	600
Facultative anaerobes	-500 ↔ +750	1250

2.7 Cell Surface interractions

While a theoretical extrapolation of biochemical processes would suggest that the outer surfaces of all bacterial cells would have a net positive charge due to the extrusion of protons and retention of electrons within the cell (VanDemark *et al.*, 1987), most bacterial cells carry a net negative surface charge, the magnitude of which is affected by the bacterial strain, the growth conditions, pH, and the presence and concentration of various inorganic molecules **(**Hancock *et al.*, 1994; Mozes and Rouxhet, 1990; Rosenberg and Doyle,1990). However, this net negative surface charge does not imply that there are no foci of positive charge present on the outer surface of bacteria (Duncan-Hewitt, 1990).

The electronic nature of the surface of bacterial cells is best described as a Guoy-Chapman-Stern layer which has a highly negative electrostatic potential wherein both divalent and monovalent cations can rapidly diffuse across the surface (Hancock *et al,* 1994). The Stern layer lies in close contact with the cell surface and is covered by a diffuse outer layer of labile ions. The thickness of this layer has been shown to be directly dependent upon the ionic strength of the adjacent electrolyte layer (Mozes and Rouxhet, 1990; Stumm and Morgan, 1996).

The interaction of a large polycation with an electrostatically charged surface will involve a localized neutralization of the negative charge of the surface layer, and ultimately results in the integration of the polycation into the outer surface of the outer membrane bilayer (Hancock *et al,* 1994). The neutralisation of this surface charge is governed by the localised interaction between the different free electrical and fixed chemical forces and results in the formation of an electrical double layer at the cell surface (Mozes and Rouxhet, 1990).

It has been shown that there is an inverse relationship between the magnitude of the surface charge and the hydrophobicity or water aversion of the adjacent structures. Since the surface charges increase the likelihood of polar interactions with the proximate water molecules, a higher concentration of these charged surface groups will correspondingly reduce the degree of surface hydrophobicity (Rosenberg and Doyle, 1990). Hydrophobicity is an interfacial phenomenon and describes the

insolubility of non-polar substances in water. Hydrophobic molecules aggregate in an aqueous environment, while hydrophilic molecules will tend to repel each other (Duncan-Hewitt, 1990).

The generation of a strongly negative electrostatic bilayer at the cell surface and the consequent exclusion of hydrophobic molecules and anionic and neutral detergents, reflects the inherent capacity of the bacterium to withstand the effects that these molecules may exert upon it. Specific interventions that serve to reduce the negative surface charge such as the addition of inorganic cations, serves to alter this surface hydrophobicity (McIver and Schürch, 1981; Rosenberg and Doyle, 1990). Bacteria that lack a high negative surface electrostatic potential are more susceptible to hydrophobic cleaning agents, biocides or antibiotics, and this has been demonstrated following the sequestration of surface associated divalent cations by means of EDTA (Hancock *et al*., 1994).

2.8 ORP and pH covariant analysis

Bacteria display an extremely diverse array of both structural and functional attributes whereby they rapidly adapt to the manifold constraints and limitations of their immediate environment. As with the response to physical gradients described in terms of temperature and pressure, bacteria are also capable of adapting to the myriad of gradients imposed by the concentrations of macro- and micronutrients, water, oxygen, carbon dioxide, hydrogen ion concentration (pH) and free energy (ORP). Thus the environmental profile required by each organism for unimpeded growth can best be described in terms of an assimilation of the individual values which together describe a distinctive range within which general growth would be supported (VanDemark and Batzing, 1987; Kimbrough *et al*., 2006).

When the relationships between the parameters responsible for growth are described in a multidimensional covariant plot, the resulting assimilation will detail the ranges of tolerance to those environmental conditions under which the bacteria are most likely to grow. It is no coincidence that the ranges of functional adaptability of the multiple biochemical and physiological processes of an optimally viable bacterium

are intimately aligned to the ranges of the physicochemical features that constitute its immediate environment.

When a sample of polymorphic prokaryotes were cultured in an artificial mediumwhere the pH and ORP values of the medium has been manipulated to describe correlates across the full theoretical range, the resultant covariant plot detailed the limits of both pH and ORP values under which bacterial viability of the sample could be sustained. The basic composition of the culture media was specifically designed to be representative of the optimal condition under which normal growth is maintained. The extreme covariate values of pH and ORP were derived from specific chemical manipulations of the media using a variety of biocompatible organic acids and buffers and excluded any processes or agents which would have resulted in the generation of any biocidal or equivalently cytotoxic compounds. Figure 1 demonstrates the range of covariant pH and ORP values which are compatible with the conditions for growth for a given bacterial population. The curve represents the extreme covariant values for both pH and ORP beyond which no growth was detected.

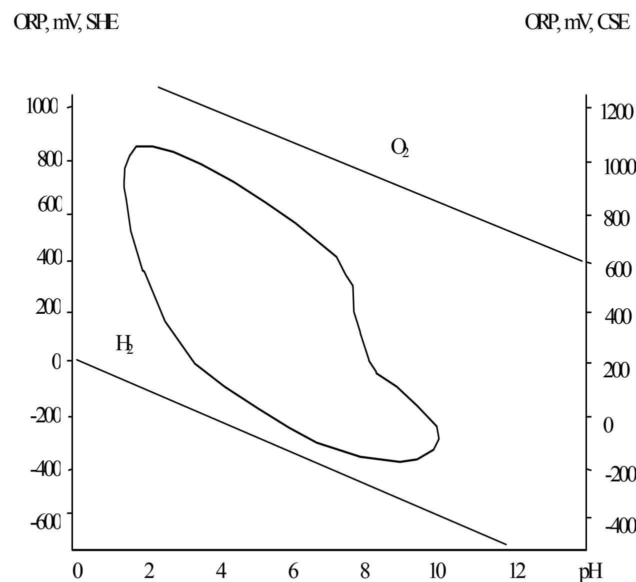


Figure 1. Range of pH and ORP values capable of sustaining microbial growth in artificial media (Prilutsky and Bakhir, 1997).

Between the pH values of 3 and 8, the microbes were able grow under a relatively broad ORP range (≤850mV). However, when the pH values approached the extremes of acidity (pH=2–3) or alkalinity (pH=8–10), the ranges of ORP values capable of sustaining vegetative growth was significantly reduced to less than 100mV (Prilutsky and Bakhir, 1997). The ranges of growth potential described by the limits in both pH and ORP for a variety of different microbial types is summarised in Table 2.

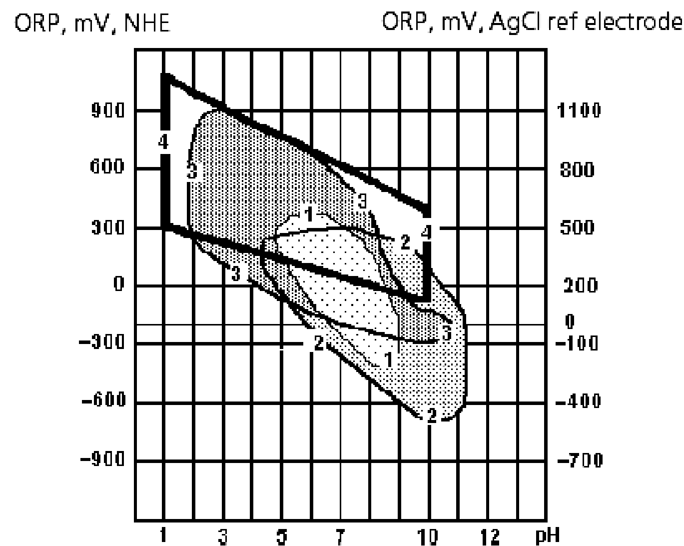
Table 2. Correlation between pH and ORP and the measure of the range of ORP values tolerated for growth of different microbial types (Prilutsky and Bakhir, 1997).

Microbial type	pH range	ORP limits (mV)	ORP Range (mV)
Acidophile	2-3	+400 ↔ +1000	600
	4-5	+100 ↔ +950	850
	7-8	-130 ↔ +820	690
Alkalophile	9-10	-120 ↔ -50	70

This data should be correlated with the reported growth limits described for bacteria of differing respiratory profiles and the ORP ranges in which they occur (VanDemark and Batzing, 1987).

As an extension to this theme, when the vitality of isolated organisms of disparate origin were assessed under varying regimes of pH and ORP, distinctive and differing ranges of survival could be detailed for each of the different organism types. When the areas of vital activity and growth of bovine sperm, *Euglena viridis* and a population of polymorphic bacterial cells were plotted on the two dimensional co-variant axes, it was possible to differentiate the specific pH and ORP ranges under which vital activity of the different organism types could be supported (Fig 2). The superimposed contour outlined by the straight bold lines (4), corresponds to the area of pH and ORP coordinates derived from a range of ECA anolyte types. The upper border of contour line 4 reflects the ORP correlate associated with anolytes of high brine mineralization while the lower border represents the ORP: pH coordinate plots for anolytes of low mineralization. Contour 2 delineates the area within which the mobility and normal shape of the Euglenas were maintained. While the area of

optimal activity of the *Euglena* cells described a trend towards high pH and low ORP values, exposure to values outside of this contour resulted in the cells becoming progressively more immobile, losing their flagellae and undergoing spherulization (Prilutsky and Bakhir, 1997).



Legend: NHE – Normal Hydrogen electrode, AgCl – Silver Chloride reference electrode

Figure 2. Covariant combinations of pH and ORP values describing the ranges of survival of isolated cells types. (1) bovine sperm; (2) *Euglena viridis*; (3) polymorphic bacteria and (4) pH and ORP values of Electro-Chemically Activated (ECA) Anolyte solutions (Prilutsky and Bakhir, 1997).

It is evident from the different contours in figure 2, that the polymorphic bacteria are significantly more resistant to reductions in pH and increases in ORP values than that of bovine sperm cells (Prilutsky and Bakhir, 1997; Prilutsky, 1999). While the overall study describes the range of environmental conditions under which microbial growth is possible, it is also a refection of the under-emphasised role that ORP plays in the maintenance of the functional and physiological integrity of the sub-cellular metabolic processes and molecular integrity.

Aside from reiterating the current perceptions that pH limits have upon bacterial growth, the covariant assessment approach introduces a new dimension to the role that the environmental ORP plays in controlling microbial growth and as a corollary, offers a means for refining antibacterial control strategies (Prilutsky and Bakhir, 1997).

2.9 Conclusions

Biological phenomena depend upon the dictates of available energy and the manner in which it is conserved.

The combination of individual atoms into stable and functional physiological structures is as a direct consequence of the energetic stability of their association. While in no way static, these separate molecular structures interact within a complex metabolic matrix to provide a platform for the creation of a vital and self sustaining organism. The intricate and fragile nature of these inter-molecular associations confers a tenuous resilience in the face of inconsistent thermodynamic forces and the persistent cooperativity between the physiologically distinct entities harmonise to produce a viable organism with unique attributes, capabilities and consequent identity.

Further to the primary energetic integration of the basic building blocks, is the requirement to maintain the functionality and physiologic interdependence of the diverse array of processes and reactions that underwrite the capacity to remain viable in a constantly variant and oftentimes stressful environment. Adaptation to a variety of physical and chemical deviations outside of the range of conditions commensurate to optimal growth remains the hallmark of the bacterial kingdom. This has resulted in the evolution of a diverse array of compensatory mechanisms which has favoured the selection of competent phenotypes and genetic templates with the potential to withstand these adverse conditions.

Given that bacterial survival is substantially premised upon energetic homeostasis, due consideration of the importance of the pivotal role of environmental oxidation-reduction potential on the same, is suggested. Aside from affording an insight into the mechanisms responsible for sustained microbial viability, manipulation of the ORP of the microbial milieu is proposed to offer a new avenue for reliable and effective control of microbial populations.

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Chapter 3

Mechanisms of biocidal action

3.1 Introduction

All bacteria maintain a substantial physiological armoury with which to withstand the adverse impacts of deviant environmental conditions. Limits to the magnitude of the capacity and the adaptability of these defensive resources in the face of extreme exposure to noxious agents may irreversibly compromise the viability of the bacterium. Notwithstanding shifts in nutrient availability or alterations in physical growth conditions, bacteria are continuously exposed to chemical compounds which may adversely impact upon their intrinsic capacity to maintain optimal physiological functionality.

There is a vast body of data that attests to the effects of a diverse range of chemical compounds on bacterial growth, but as with the exquisitely complex matrix of molecular interactions that govern bacterial survival, so to, there is an equally complex and challenging milieu of physicochemical relationships that need to be embraced in order to achieve sustainable bacterial control. The response of a bacterium to an adverse condition or agent will depend upon a number of factors which will include the organism type, the nature of the agent itself, the intensity of the insult, and the duration of the exposure to the same. In addition to these factors, other features such as the stage of the growth cycle, the presence of intrinsic and acquired mechanisms to withstand stress, largely theoretical extrapolations from field conditions, as well as laboratory technique, will all influence the interpretation of the requirements for the development of a consistently reliable bacterial control strategy.

Antibacterial agents are broadly categorised into those factors derived from natural processes and those that are artificially synthesised. In the former, factors that dictate the physical and chemical environment of the bacteria i.e temperature, pressure, nutrition, oxygen concentration and sunlight all exert a direct influence on bacterial growth. In terms of exposure to chemical agents, natural compounds with the capacity to cause bacteriostasis and/or a bacteriocidal effect are generally the 'true'

antimicrobials or antibiotics, and expanded production of these compounds has been refined and expanded into industrial chemical syntheses. These refined compounds are essentially selective in their mechanism of action and their dose and exposure requirements can be targeted at the specific bacterial population. This application optimisation has the benefit of minimising any adverse side effects to the host and the environment.

The category of chemical agent with the broadest impact across the maximum range of all bacterial types and application conditions is loosely described as being biocidal. This extended range of antimicrobial capacity has given rise to performance based descriptions which include antiseptics, disinfectants, sanitisers, preservatives, bacteriocides and sterilants. For the purposes of this study, the term ‘biocide’ will be used to detail and describe those chemical agents responsible for the strategic control of environmental bacterial populations. Chemical biocides are further categorised according to their composition, their mode of action and their field of application. This classification broadly differentiates between non-oxidising and oxidising biocides, but also includes unrelated compounds such as surfactants and chelating agents that will influence the outcome of the biocidal intervention.

3.2 Biocidal effects of physical agents

While the mechanism of action of temperature and radiation may appear somewhat unrelated to the biocidal effects of conventional chemical agents, the effects of both agents should be viewed from an energetic perspective wherein the metabolic disruption that ensues following an excessive insult appear to parallel the changes induced by exposure to chemical biocidal agents. It has been shown that radiation induces the intracellar formation of singlet oxygen, superoxide and peroxide anions as well as other highly reactive molecular and ionic species. These elements are highly detrimental to cells and aside from specific alterations to the DNA molecule they also result in generalised oxidation damage to other essential cell components (Caldwell, 1995).

3.3 Biocidal effects of chemical agents

In terms of classifying the diverse array of chemical biocides, it is useful to consider the range of compounds both in terms of their specific mode of action as well as the chemical characterisation of the compounds themselves.

The general modes of action of antimicrobial chemical agents comprise the following:

1. Inhibition of enzyme activity,
2. Inhibition of nucleic acid function,
3. Disruption of cell wall formation and function,
4. Inhibition of cell wall synthesis, and
5. Alteration of membrane function (Caldwell, 1995, Russell, 2001).

Aside from the different sites of biocidal activity, the impact of chemical biocides on microbes can be further classified according to the component of the cell where the specific compound exerts its effect.

3.3.1 Cell walls

The physicochemical and energetic relevance of the different components of the various barrier structures have been discussed earlier, and it was noted that the distinctive features of the vegetative gram positive and negative bacterial cell types each confer specific intrinsic antibiocidal attributes that require differentiated control strategies. Bacterial spores with a protective coat are recognised to be metabolically inactive. This oxidised or electron deficient state, results in a tendency for the acceptance of electrons whereby they becoming reduced, and thus protected by reducing agents. Conversely, metabolically active vegetative cells readily donate electrons from the transitional metals embedded in the cell wall surface and become progressively more oxidised. However the quantitatively finite nature of the mechanisms to counter an extended exposure to an oxidative stress will result in cell death (Marnett, 2000; Russell, 2001).

3.3.2 Cytoplasmic membrane.

As a ubiquitous and critical component of all bacterial cells, damage to the cytoplasmic membrane may result from a change in composition, fluidity, structural organisation and/or electronic charge. The effects that follow biocidal damage include the disruption of enzyme and transport activities, the abolition of energy generating capacity and the leakage of critical intracellular materials, all of which will result in the destruction of the morphological and physiological integrity of the cell (Caldwell, 1995; Russell, 2001).The leakage of cellular contents is not a primary effect but is rather a consequence of the disruption of the transmembrane electrochemical proton gradient as well as the uncoupling of the associated oxidative phosphorylation process (Russell, 2001; Helbling and VanBriesen, 2007). The uncoupling of oxidative phosphorylation refers to the dissociation of oxidation from phosphorylation, which results in a rapid backflow of protons into the cell and the ultimate collapse of the proton motive force. It is the inability to maintain the energy based electro-osmotic gradient across the membrane which results in the leakage of cellular contents to the outside (Russell, 2001).

While it is predominantly the non-oxidising biocides that have been reported to impact upon the permeability of the cytoplasmic membrane i.e. phenols, Quaternary Ammonium Compounds (QAC's), alcohols and biguanides (Russell, 2001), any compound that uncouples the oxidative phosphorylation capacity of the membrane and thereby destroys the transmembrane proton gradient, will result in a loss of sustainable membrane integrity and consequential leakage of cytosolic constituents.

3.3.3 Nucleic Acids

Aside from the direct energetic effects of radiation, other energy based agents may play a role in disrupting nucleotide functionality. While the impact of most biocides will result in changes to the cell barrier system, alterations at this level inevitably translate into autolytic metabolic disturbances within the cytoplasm. The deviant metabolites elaborated from inappropriate or incomplete reactions have been shown to act as endogenous genotoxins to the DNA strand (Fridovich, 1979; Thomas and Aust, 1986; Marnett, 2000).

Interference with the DNA molecule can be physiologically devastating to the cell and the adverse changes will include:

1. Structural interference resulting in strand separation
2. Intercalation or incorporation of false residues, and
3. Physiological interferences which impact upon the DNA polymerase enzymes (Marnett, 2000)

Russell (2001) has also reported on the inhibition of DNA synthesis which results from cationic ionization as well as the strand breakages which are associated with peroxide treatment, however these genotoxic changes have also been shown to include the consequent dysfunction allied to the alkylation and intercalation of polycyclic planar molecules that will distort the DNA helix and result in frame shift mutations and critical code changes (Caldwell, 1995).

3.4 Chemical classification of biocides

Biocidal agents are derived from a diverse array of chemical compounds, but the basic classification will be restricted to an interpretation of their mode of action. Given the fundamental energy based theme that has been developed in the discussion thus far, the compounds will be differentiated as being either Non-oxidising and Oxidising in their mode of action.

3.4.1 Non-Oxidising Biocides

These compounds differ substantially in their respective modes of action, but all share a similarity in that they are all non-oxidising organic compounds. The biocidal activities vary from direct disruption of the cell wall and outer membrane structures (detergents, QAC's, biguanides, phenols), intracytoplasmic disruption (QAC's biguanides, aldehydes, phenols) and cytoplasmic membrane damage (phenol derivatives) (Denyer and Stewart, 1998; Russell, 2001; Cloete, 2003). The substantial overlap in terms of site of action does not reflect upon a definitive description of the primary site of biocidal activity to the exclusion of the effects of secondary events initiated by the initial insult (Denyer and Stewart (1998).

3.4.2 Oxidising biocides

In accordance with their physicochemical composition, oxidising biocides exert their biocidal effect on the basis of their thermodynamic status. Their electron deficient state confers a heightened reactivity and the compounds act as scavengers of bacterial associated energy. However, these compounds are substantially non-selective and will react on a gradient of optimal thermodynamic efficiency with any source of oxidisable material. Effective biocidal control strategies with these reagents thus require an appropriate understanding of the REDOX profile of the total bacterial environment.

The three categories of oxidising biocides with relevance to energy based antimicrobial control are the oxidising halogens, the peroxides and oxygen derivatives.

3.4.2.1 Chlorine

3.4.2.1.1 Basic Chlorine Chemistry

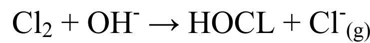
Chlorine was discovered in 1774 by Carl Scheele, but it was only in the early 1800's that it was specifically employed as a biocidal intervention.

The oxidising capabilities of chlorine can best be demonstrated when Cl_2 is seen to comprise of two chlorine atoms of opposite charge i.e. $Cl^{+1}Cl^{-1}$. In order to cause the dissociation of molecular chlorine (Cl_2) it is necessary for the Cl^+ atom to acquire two electrons and become reduced to 2 x Cl^- (White, 1992, Stumm and Morgan, 1996).

Due to the valency of molecular chlorine which ranges between -1 and +7, it is capable of forming a complete series of oxyacids which range from HClO to $HClO_4$ (White, 1992). When chlorine is added to water at neutral pH, hypochlorous acid and hypochoric (hydrochloric) acid are produced.

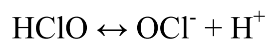
$$Cl_2 + H_2O \rightarrow HOCl + HCl\ (H^+ + Cl^-)$$

The halogen chemistry of chlorine and its aqueous derivatives is a highly dynamic system and the diverse array of potential reactions are substantially dependent on a variety of factors of which pH has been reported to be the most important (White, 1992; Stumm and Morgan, 1996). When chlorine is added to water with a pH of less than 3, the predominant reactive species will be chlorine gas.



$$Cl_2 + OH^- \rightarrow HOCL + Cl^-_{(g)}$$

The highly reactive nature of hypochlorous acid in water of neutral pH, results in the spontaneous dissociation into its hypochlorite anion with release of a hydrogen ion.



$$HClO \leftrightarrow OCl^- + H^+$$

The concentrations of hypochlorous acid and hypochlorite are near equivalent at neutral pH and a reduction in pH shifts the reaction towards hypochlorous acid (optimum 3.5 – 5.5), while alkalinising the solution pushes the reaction towards hypochlorite production (Fig 1).

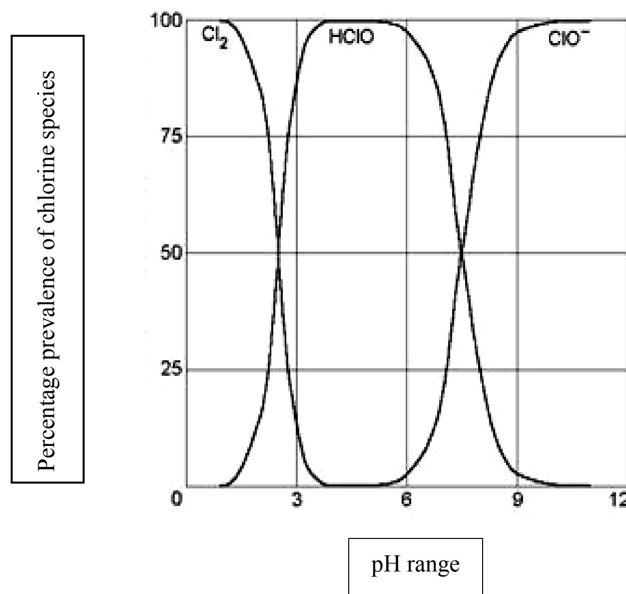


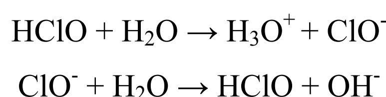
Figure 1. Prevalence of chlorine and oxy-chlorine species in aqueous solution as a function of pH. (Bakhir *et al*., 2003)

This relationship in confirmed by the relative proportions of hypochlorous acid and hypochlorite anion found in solution over the extended pH range (Table 1) (Rowe, 2001; Eifert and Sanglay, 2002; Parish et al., 2003; Sapers , 2006, Guentzel *et al*., 2008).

Table 1. Relationship between the relative proportion of hypochlorite ions and hypochlorous acid in solutions over different pH values.

pH	HClO (%)	ClO^- (%)
6.5	92	8
7.0	79	21
7.5	55	45
8.0	28	73
8.5	11	90
9.0	4	96

It has been reported that the Oxy-chlorine compounds have the highest bactericidal activity at a pH 7.5 - 7.6 where the hypochlorous acid and hypochlorite moieties are in equivalent ratios. At this pH range the conjugate acid-base pair reaction is as follows:

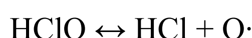


$$HClO + H_2O \rightarrow H_3O^+ + ClO^-$$

$$ClO^- + H_2O \rightarrow HClO + OH^-$$

Under these conditions, the primary oxy-radicals are capable of generating further metastable radicals whose biocidal activity far exceeds that of the parent hypochlorous acid. These reactive species include singlet molecular oxygen ($^1O_2$), hypochorite radical (ClO·), chlorine radical (Cl·), atomic oxygen (O·) and hydroxyl radical (OH·) (Bakhir *et al*. 2003).

In addition, hypochlorous acid may also dissociate into hydrochoric acid and the highly reactive molecular oxygen radical (White, 1992).



$$HClO \leftrightarrow HCl + O\cdot$$

While Chang in 1944 somewhat prematurely dispelled the belief that it was the nascent oxygen liberated during the dissociation of the hypochlorous acid to hydrochloric acid and singlet Oxygen that was responsible for the germicidal action of hypochlorous acid (White, 1992), it is now recognised that the role of Reactive Oxygen Species (ROS) and other hydroperoxi-radicals arising from a biocidal insult, that are fundamental to the ensuing secondary and largely irreversible cellular dysfunction.

3.4.2.1.2 Mechanism of Action

The exact mechanisms involved in the elimination of bacteria by free chlorine compounds have not been fully elucidated (Kim *et al.*, 2000; Helbling and VanBriesen, 2007), but it has been proposed that the predominant reaction involves the oxidation of the bacterial membrane which through an increase in permeability results in the leakage of macromolecules and ultimately cell death.

Recent studies have shown that the main mechanism of inactivation in response to oxidative stress is more subtle, and relates to the uncoupling of the electron chain with strategic enzyme inactivation (White, 1992, Kim *et al.*, 2000, Helbling and VanBriesen, 2007). This assertion is supported by the close correlation between the oxidation of the sulfhydryl groups of proteins and enzymes and the overall mechanism of antibacterial action of Chlorine based compounds (Thomas, 1979, Park *et al.*, 2004).

3.4.2.1.3 Free Chlorine

Depending on the determinants of the solution, i.e. pH, temperature etc, aqueous chlorine is present in a range of reactive forms, and it is necessary to differentiate between these categories in order to formulate a predictable biocidal effect.

The total chlorine in a system equals the 'Free chlorine' plus the 'combined chlorine'. Free or active chlorine refers to compounds which include Cl_2, HOCl and ClO^-, while combined chlorine refers to chlorine in combination with Ammonia (Chloramaines) and other nitrogenous or 'N-Chloro' compounds (Stumm and Morgan, 1996). The available chlorine relates to the concentration of hypochlorous acid and hypochlorite ions that are present in chlorinated water, and as a measure of the oxidising power of the solution, it reflects the quantity of chlorine that is capable of releasing an equivalent amount of reactive oxygen. Free chlorine is measured by iodometric titration, and its accuracy is dependent upon the sensitivity of the assay to exclude the reactivity of non-chloroxy based compounds which may bias the results (White, 1992).

3.4.2.1.4 Chlorine demand

The projected efficacy of any chlorine based biocidal intervention requires an in depth assessment and understanding of the factors that will influence both the qualitative and quantitative availability of the reactive oxidant species required for the minimum biocidal effect. These physical factors include pH, temperature, conductivity, turbidity, total organic carbon, total chlorine, combined chlorine and free chlorine (Helbling and VanBriesen, 2007).

In addition, the rational choice of a chlorine based biocidal compound requires a well considered insight into the capability of the target microbial population to withstand the oxidative stress. Therefore a more holistic understanding of the prevailing microenvironmental conditions is required in order to refine the type, rate and frequency of oxidant biocide exposure that would be required for optimal bacterial control. The chlorine demand of a bacterial suspension is described as the difference between the initial chlorine concentration and the residual chlorine concentration subsequent to exposure. It is recognised that it is the free chlorine component that reacts with the widest range of bacterial contaminants, and it has been demonstrated that the ultimate chlorine demand is directly proportional to the measure of ultimate bacterial cell survival (Helbling and VanBriesen, 2007). Additionally, the presence of non-microbial reductants in the form of both inorganic and organic materials, as well as the overall bacterial bioload of the system will impact on the likely efficacy of the chlorine based intervention. With progressive exposure, chlorine demand will eventually stop and this reflects the condition where all organic material that was originally present and available for reaction with chlorine has been exhausted.

Helbling and VanBriesen (2007) reported that the degree of sensitivity to oxidative stress can be calculated according to the chlorine demand and demonstrated that the chlorine contact time for a 3-log inactivation of pure culture suspensions of *Escherichia coli, Staphylococcus epidermidis* and *Mycobacterium aurum* was 0.032 ± 0.009, 0.221 ± 0.08 and 42.9 ± 2.71 mg min/l respectively. The elevated chlorine demand by *M. aurum* has been proposed to relate to the high concentration of mycolic acids in the mycobacterial cell wall. This feature has been suggested to be a contributing factor to the substantial resistance of *Mycobacteria* spp. to free chlorine,

antibiotics and other disinfectant compounds that has been reported (Best *et al*., 1990; Sattar *et al*., 1995; Helbling and VanBriesen, 2007).

Aside from the species specific physical attributes that facilitate tolerance to chlorine residuals, bacteria also defend themselves against oxidative stress by both inherent and adapted resistance mechanisms that result in the production of extracellular polymeric substances or EPS. The presence of EPS has been shown to progressively reduce the concentration of the disinfectant that ultimately becomes available at the cell wall or membrane surface (Brözel, 1992; Brözel and Cloete, 1993, Cloete, 2003). It has been demonstrated that resistant organisms exert a chlorine demand well in excess of sensitive organisms while still remaining viable (Helbling and VanBriesen, 2007).

Chlorine demand displays a linear relationship to the initial free chlorine concentration, and while the elevated demand associated with an initial high chlorine concentration is predominantly due to the oxidation of inactive cellular material, the persistent demand relates to ongoing oxidation of leaked intracellular macromolecules and other oxidative intermediates initiated by the biocidal intervention.

While the use of free chlorine sensors have been proposed as a plausible surrogate monitor to assess the degree of bacterial inactivation, the inability to factor in the effects of independent variables such as variations in bacterial susceptibility as well as evolving resistance trends, has constrained the universal adoption of this approach (Helbling and VanBriesen, 2007).

3.4.2.2 Oxy-chlorine products

3.4.2.2.1 Hypochlorous acid (HOCl):

Given the dynamic nature of the constituents of a chlorinated solution, the presence of the substantially labile hypochlorous acid (HOCl), is primarily due to the the effects of pH manipulation. HOCl generally requires on-site production and only predominates in solution when the pH range is fixed between 5 and 7.5. Aside from on-site electrochemical generation, hypochlorous acid can also be produced from

aqueous calcium hypochlorite using a pH adjustment with hydrochloric acid (Mokgatla *et al*., 2002).

At a neutral pH, the hypochlorous acid fraction is equivalent to the “free available chlorine residual” and the scale of reactivity or oxidising power relative to the other chlorine compounds in solution can be described as follows:

$HOCl > Cl_2 > OCl^-$ (White 1992, Rowe, 2001).

The biocidal efficacy of HOCl has been attributed to the relative ease with which the molecules can penetrate bacterial cell walls. Due to its similarity in size to water as well as its electrical neutrality (White, 1992), it has been suggested that HOCl gains access to the periplasmic space directly through the barrier porins and that its passage is not impeded by steric hindrance, electrostatic repulsion or by blocking as may occur with larger molecules traversing the LPS monolayer (Mokgatla *et al*., 2002).

3.4.2.2.2 Hypochlorite anion (OCl^-)

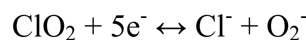
Due to its anionic charge, the hypochlorite anion displays restricted capacity to diffuse through the cell wall. It appears to act on surface proteins by disrupting the transport of solutes, and thereby disturbs the cellular osmotic balance. It has also been reported to oxidise the sulfhydral groups of proteins and to inhibit the plasma membrane ATPases. The predominance of this species in alkaline conditions and its prescribed biocidal range can be directly linked to a parallel reduction in the free available chlorine concentration. It has been reported that the limited biocidal efficacy of hypochlorite at pH values of 9 and greater is due to the conversion of up to 96% of the active chlorine into non-oxidant species which include chloride, chlorate and perchlorate (White, 1992).

Additionally, the notion that the mere presence of a chlorine based compound will confer a biocidal effect discounts the significance of the reactivity or REDOX status of the compound. This effect was elegantly illustrated where the elimination of a culture of *B. anthracis* with a solution containing an active chlorine concentration of 50ppm took 40 minutes at a pH of 8.6, while the elimination of the equivalent culture

with the same free chlorine concentration but adjusted to pH 7.2, required only 20 minutes exposure (Bakhir *et al*., 2003b). It has also been shown that hypochlorite anion is up to 80 times less efficacious than hypochlorous acid at an equivalent concentration, and this disparity is further accentuated at increased temperatures where the additional energy drives the dissociation of HOCl to H^+ and OCL^- (White, 1992).

3.4.2.2.3 Chlorine Dioxide (ClO_2)

Chlorine dioxide is a highly selective oxidant and reacts most readily with compounds that easily donate an electron (Stumm and Morgan, 1996)**.** On a strictly molecular weight basis, the ratio of Chlorine (Cl_2 = 35.45) relative to that of chlorine dioxide (ClO_2 = 67.45) equals 1.9. Thus, theoretically 1.9mg of ClO_2 is equivalent in oxidizing power to 1mg of Chlorine. However since the chlorine moiety of chlorine dioxide is 52.6% by weight, and as it requires five valence changes in order to become reduced to Cl^-, this equates to a 263% difference in available chlorine oxidizing power relative to that of Cl^-. The following equation describes this reaction.



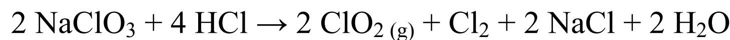
$$ClO_2 + 5e^- \leftrightarrow Cl^- + O_2^-$$

The availability of this oxidising power is strongly pH dependent, and at a neutral pH, ClO_2 becomes reduced to Chlorite (ClO_2^-) with 1 valency change, while at pH = 2, it is reduced to chloride with 5 valency changes. Hence at neutral pH, ClO_2 only exhibits 20% of its full oxidizing potential. Conversely ClO_2 is substantially more germicidal than Cl_2 at a pH of 8.5 where the Cl_2 - HOCL residual is 89%, than at a pH of 6.5, where the Cl_2 – HOCL residual has been reduced to 8.7%. Additionally ClO_2 will not hydrolyse with water as is the case with Cl_2 but it displays a high solubility in especially chilled water (White, 1992).

Despite the claim that ClO_2 does not react with nitrogenous compounds, it still exhibits a higher chlorine demand than Cl_2 during the treatment of waste-water. White (1992) has reported that ClO_2 has a more rapid coliform inactivation rate relative to that of Cl_2. However, in all cases, the magnitude of the final “kill” rate with Cl_2

exceeded that of ClO_2, and this highlights the spontaneous as opposed to latent oxidative capacity of the two compounds.

Historically Chlorine dioxide was produced by the reaction of Sodium chlorate with sulphur dioxide to produce chlorine dioxide and sodium sulphide. Alternatively, the highly explosive gaseous form of ClO_2 can be generated by the following reaction:



$$2\ NaClO_3 + 4\ HCl \rightarrow 2\ ClO_{2\ (g)} + Cl_2 + 2\ NaCl + 2\ H_2O$$

Aside from the generalised oxidative disruption to both cell wall and membrane integrity, ClO_2 also impacts upon protein function through the destruction of RNA with resultant disruption of protein synthesis. While it has been claimed that the reactivity of ClO_2 does not promote the formation of Trihalomethanes (THM), most ClO_2 generators still produce Cl_2 which will result in THM formation. In addition it also results in the production of other Disinfection bi-products (DBP) of which both chlorates and chlorites have been shown to be hazardous to mammals (White, 1992).

3.4.2.2.4 Chloramines:

Chloramines are combination products and arise from the association of mainly hypochlorous acid with both inorganic nitrogen compounds eg. ammonia (NH_3), nitrites (NO_2^-) and nitrates (NO_3^-), as well as organic nitrogen molecules which comprise amino acids and proteins. These nitrogenous compounds skew theoretical chlorination equations by reducing the availability of free chlorine. As with all chloroxy-based compounds, these reactions are also pH dependent. It has been shown that monochloramine formation is favoured at pH 8, dichloramine at pH 5, and that trichloramine predominates at a pH of less than 5 (White, 1992). Chlorine will also combine with the nitrogenous components of bacteria forming chloramines and chloramides. Chloramine production exceeds that of chlorohydins at low levels and describes a directly dose dependent conversion relative to the HOCl concentration (Carr *et al*., 1998; Spickett *et al*., 2000). The action of chloramines has been reported to display a close correlation between the oxidation of bacterial sulfhydryl bonds and overall bactericidal effect. This study showed that the progressive reduction in chloramine compounds described a direct relationship with an increasing degree of

oxidation of bacterial sulfhydryl bonds, and paralleled the concomitant loss of microbial viability (Thomas, 1979).

A saturated chlorine demand is reported to reflect an exhaustion of free available hypochlorous acid, and the increasing oxidising equivalence of the low molecular weight endogenous chloramines and their derivatives appear to perpetuate the sulfhydryl oxidation and peptide fragmentation that ultimately results in the cell death (Thomas, 1979).

3.4.2.3 Bromine Compounds

While Hypobromous acid (HOBr) is a weaker oxidant relative to hypochlorous acid, both hypohalous acids react in a similar manner against an array of biological molecules such as thiols, thiol-esters, amines, amino acids and unsaturated membrane lipids. These unsaturated lipids play a critical role in optimal DNA-membrane interactions that are necessary for bacterial replication (Carr *et al*., 1998).

Relative to the halochlorines, hypobromous acid displays a predilection for membrane associated unsaturated phospholipids (Spickett *et al*., 2000) and the resultant formation of bromohydrins exceeds that of chlorohydrins by a 10 fold measure under equivalent conditions. While hypobromous acid also results in the production of bromamines, this reaction is strictly secondary relative to that of chloramine production by hypochlorous acid (Carr *et al*., 1998, Spickett *et al*., 2000). Notwithstanding, the heightened biocidal capacity of bromamines relative to chloramines has been reported to be due to their greater reactivity in terms of secondary oxidative reactions that they induce (Carr *et al*., 1998). In the presence of other oxidising biocides and especially ozone, reactions with bromine compounds result in the formation of hazardous bi-products such as bromate which limits their application in human contact uses.

3.4.2.4 Peroxides

The peroxides are unstable oxygen compounds which decompose to form free hydroxyl radicals. These compounds readily react with organic compounds. The

peroxides include hydrogen peroxide, peracetic acid, aromatic peroxyacids, persulphates and calcium peroxide.

3.4.2.4.1 Hydrogen Peroxide (H_2O_2)

The ideal biocide is proposed to encompass the following attributes, in that it:

- will be effective against micro-organisms when highly diluted
- will be low in toxicity to people and animals, and
- will not injure the environment (Block, 1991).

When employing H_2O_2 as a sanitiser, the relative effects of conventional halide based products with an equally rapid disinfection action are readily superceded. Hydrogen peroxide is totally miscible with water and readily penetrates cells causing site-directed damage due to the metalo-dependant hydroxyl formation. The antimicrobial action of H_2O_2 is proposed to be due to its oxidation of sulfhydryl groups as well as the double bonds in proteins, lipids and surface membranes (Block, 1991). In contact with DNA, H_2O_2 causes strand breaks due to the hydroxylation of the Guanine and Cytosine nucleotide bases. However, hydrogen peroxide is routinely produced in cells by the reduction of oxygen. As a critical oxidant stressor, H_2O_2 activates a variety of regulatory genes which modulate the intracellular redox potential. In a direct response to this noxious threat, all cells have evolved a variety of genetically encoded cellular defence mechanisms which comprise superoxide dismutases to scavenge superoxide, catalases, alkyl hydroperoxide reductases and glutathione reductases to scavenge hydrogen peroxide, as well as a variety of DNA repair enzymes to counter its presence and further catalytic activity (Fridovich, 1978, Fridovich,1979; Block, 1991).

Hydrogen peroxide generally displays a greater degree of biocidal efficacy against gram negative than gram positive bacteria, and anaerobes display particular sensitivity due to the absence of the catalase enzyme which converts the peroxide to water. The biocidal action of H_2O_2 is not pH sensitive, but a heightened sporicidal efficacy due to increased protein extraction from the spore coat has been reported under acidic

conditions. Additionally, the activity of hydrogen peroxide is reported to be synergistically enhanced by the presence of iron and copper salts (Block, 1991).

When hydrogen peroxide decomposes, the formation of hazardous bi-products are obviated as only oxygen and water are evolved. It displays broad spectrum antimicrobial ctivity and has extremely low environmental toxicity.

3.4.2.4.2 Organic peroxides - Peracetic acid

Peracetic acid or the peroxide of acetic acid has the same antimicrobial attributes of hydrogen peroxide. As a weak acid it displays greater activity at an acid pH, and the residual components comprise acetic acid, hydrogen peroxide, water, oxygen, and dilute sulphuric acid. Peroxiacetic or peracetic acid is not inactivated by bacterial catalase or peroxidase enzymes and hence is a more potent antibacterial agent than hydrogen peroxide. Peracetic acid has been shown to be sporicidal at low temperatures and it retains its biocidal activity in the presence of organic material. As with hydrogen peroxide, it forms free hydroxyl radicals which react with various lipid and protein structures and DNA (Block, 1991).

3.4.2.5 Oxygen Radicals

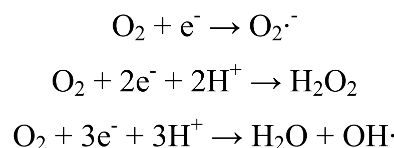
While the descriptions of the abovementioned commercial biocides have attempted to prescribe the causal relationship between the changes to bacterial cells and the targeted biocidal exposure, the nett effect of the primary intervention may not necessarily reflect the consequential cellular damage that occurs largely secondary to the initial toxic insult.

In order to refine the measure of predictability of any biocidal intervention, it is necessary to recognise the exquisitely delicate balance that characterises the optimally homeostatic biochemical milieu of the bacterial cell. Aside from withstanding targeted biocidal control strategies, bacteria persist and in some cases flourish in the presence of continuous and substantially adverse chemical and physical onslaughts.

As detailed by Fridovich (1979), oxygen, as the critical component of aerobic respiration, like Janus has two faces - one benign, and the other malignant. While the aerobic lifestyle offers great advantages, it is also fraught with danger, and it is well known that molecular oxygen and its reactive metabolites are toxic to all life forms (Fridovich, 1978, Marnett, 2000). The delicate balance that underlies normal physiological metabolism is reinforced by the fact that all living organisms are thermodynamically unstable with respect to the oxidation by molecular oxygen or dioxygen (Hill, 1979).

Kimbrough *et al.*, (2006) have reported that the addition of dissolved oxygen to a contaminated water medium resulted in distinctive shifts in the ORP which in turn caused alterations in the metabolic profile of the microbe resulting in the suppression of growth. While intrinsic to aerobic respiration, the seemingly innocuous reduction of oxygen is also capable of producing the superoxide radical ($O_2\cdot^-$), Hydrogen Peroxide (H_2O_2), Hydroxyl radical ($OH\cdot$) and singlet oxygen ($^1O_2$). The hydroxyl radical is said to be strongest biological oxidant yet known**,** and readily attacks membrane lipids, DNA, and other essential cell components (Fridovich, 1978). This assertion is confirmed by Bielski and Shiue (1979), who report that the hydroxyl radicals are 10 times more reactive relative to the superoxide radical.

These reactions occur as follows:



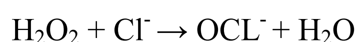
$$O_2 + e^- \rightarrow O_2\cdot^-$$

$$O_2 + 2e^- + 2H^+ \rightarrow H_2O_2$$

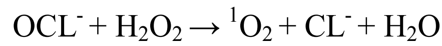
$$O_2 + 3e^- + 3H^+ \rightarrow H_2O + OH\cdot$$

This series of reactions are substantially replicated in the presence of Myeloperoxidase (MPO), a heme enzyme present in the primary lysosomal granules of neutrophils and mononuclear phagocytes. MPO also catalyses the oxidation of halide compounds by transferring electrons to the H_2O_2 to generate an oxidized halide (Mukhopadhyay and Das, 1994).

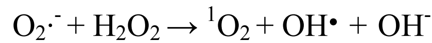
The classic MPO catalysed reactions comprises the following:



$$H_2O_2 + Cl^- \rightarrow OCL^- + H_2O$$

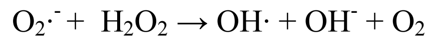


$$OCL^- + H_2O_2 \rightarrow {}^1O_2 + CL^- + H_2O$$

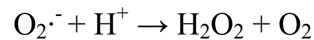


$$O_2\cdot^- + H_2O_2 \rightarrow {}^1O_2 + OH\bullet + OH^-$$

In addition superoxide radical also reacts with hydrogen peroxide to produce the hydroxyl radical, while the superoxide anion may react with bacterial SOD to produce additional hydrogen peroxide (Block, 1991).



$$O_2\cdot^- + H_2O_2 \rightarrow OH\cdot + OH^- + O_2$$



$$O_2\cdot^- + H^+ \rightarrow H_2O_2 + O_2$$

The transition metals i.e. Fe, Cu, Cr, Co and Mn are all proposed to catalyse the formation of the highly toxic hydroxyl radical by way of the Fenton and Haber-Weiss reactions.

$$H_2O_2 + Fe^{2+} \rightarrow OH\cdot + OH^- + Fe^{3+}$$

In addition to reports that have shown that chelation of these metal ions by EDTA will eliminate the antibacterial action of hydrogen peroxide (Block, 1991), transmission electron microscope images of bacteria exposed to reactive oxygen species including hydroxyl radical, ozone and peroxide, display a substantive similarity to the changes induced by the hydroxyl radical generated by the fenton reaction (Jeong *et al*., 2006).

In response to an oxidative stress, cells have developed the capacity to become more resistant to the deleterious factor within hours of exposure to sub-inhibitory quantities of the factor. Enzyme induction in the face of oxidative stress is both extremely rapid and effective. Exposure to hyperbaric oxygen resulted in an increased production of the superoxide radical and this was paralleled by a concomitant increase in SOD production. Cultures of *Escherichia coli* grown under anaerobic conditions have been shown to contain predominantly FeSOD enzyme, while the same culture shifted to the MnSOD type enzyme when exposed to oxygen. Superoxide dismutase enzymes are highly effective catalysts and have been reported to react with $O_2\cdot^-$ at a rate 2 x 10^9 M^{-1} sec^{-1} (Fridovich, 1978). Three distinctive types of SOD enzyme have been described – FeSOD and MnSOD occur in prokaryotes, while the Cu/ZnSOD type is specific to eukaryotes. Gram positive bacteria contain predominantly MnSOD, while

most gram negative bacteria, as well as the gram positive *Staphlococcus aureus* have been shown to contain both FeSOD and MnSOD (Nester, 1973). From an evolutionary perspective, it is significant to note that eukaryote mitochondrial MnSOD and bacterial MnSOD share a homologous amino-acid sequence (Fridovich, 1978).

Protection against autogenous H_2O_2 damage is afforded by the steady state induction of catalase enzymes under normal aerobic respiratory conditions, however this defence mechanism is rapidly overwhelmed when exposed to concentrations of H_2O_2 that are conventionally used for practical disinfection (Block, 1991).

3.4.2.6 Ozone

Ozone is an unstable gas with a short half life and needs to be generated at the site of application. It is a potent bactericide and virucide and is also a potent oxidant of chemical compounds including Fe^{2+}, Mn^{2+}, MnO_4, NO_2^- and CN. Ozone has a high solubility in water and contact with organic material readily causes reversion to oxygen.

Its potent biocidal properties are significantly different from those of chlorine based compounds, and this is primarily ascribed to the substantially elevated REDOX potential. Aside from the cascade of potential reactive oxygen species that may evolve from the reduction of ozone, the relatively small doses required have made it difficult to discern between the quantity initially applied and the residual quantities which are necessary for effective disinfection. As with other oxidising biocides, the mechanism of action is broadly described as being a 'lytic phenomenon' which ensues from bacterial cell wall or membrane disintegration.

In the absence of halides, the bi-products of ozonation comprise a variety of low molecular weight acids, aldehydes, alcohols and ketones, many of which retain biocidal properties in their own right (White, 1992).

3.4.3 Electric fields

Electric fields and currents have been shown to be capable of disinfecting drinking water and reducing the numbers of bacteria, viruses and yeast in food. As a non-thermal intervention, Pulsed Electrical Fields (PEF) or High Electric Field Pulses (HELP) has been studied extensively for its microbial inactivation effects (Wouters *et al*., 2001). Sterilization of contaminated water within an electrochemical cell has been achieved after a 15.7 min exposure to a 2.5 mA/cm^2 or 125 mA electrical field regardless of the initial microbial density (Drees *et al*, 2003).

This effect is confirmed by Jeong *et al*., (2006), where microbial suspensions in an electrolytic cell did not display any inactivation in the absence of the applied current. The poteniation of a variety of industrial biocidal agents by the simulataneous application of a low voltage electrical charge resulted in a complete bacterial kill (>6 $\log_{10}$) in contrast to a 1 $\log_{10}$ unit reduction when the biocide or electric current were applied independently (Blenkinsopp *et al.*, 1992).

Similarly, the application of a direct current to water has been reported to cause dramatic shifts in the oxidation-reduction potential of the medium (Kimbrough *et al*., 2006). As expected, a variety of potent chemical oxidants are also generated when an electric current is applied to an aqueous suspension of bacteria through a system of immersed electrodes. These oxidants include hydrogen peroxide, ozone, free chlorine and chlorine dioxide (Kimbrough *et al*., 2006, Pak and Chakrovortty, 2006). However it has also been demonstrated that these oxidants are not exclusively responsible for the resultant cell death that follows the application of a direct current (Drees *et al*, 2003). The mechanism of action has been ascribed to an irreversible membrane permeabilization process, a direct oxidation of cellular constituents by the electric current, as well as the biochemical oxidation due to the chemical oxidants formed during the electrolysis (Wouters *et al*., 2001; Drees *et al*., 2003). This oxidant effect has been confirmed where the inclusion of glutathione as a reducing agent resulted in a significant attenuation of the bacterial inactivation (Drees *et al*, 2003).

Cell death is proposed to be due to either the formation of permanent pores and subsequent destabilization of the cell membrane or the loss of critical components and

destruction of chemical gradients across the membranes. These electrically induced pores arise as a result of a process termed Electroporation (Wouters *et al*., 2001). Electropermeabilisation refers to the formation of transient pores in the membrane and is a function of the magnitude of the induced transmembrane potential, as well as the duration of the exposure to the external electric field (Wouters *et al*., 2001; Drees *et al*., 2003). The application of a transmembrane potential exceeding 1 V for an extended pulse time (>10min), will lead to irreversible membrane permeabilization and cell death (Drees *et al*, 2003).

Studies on artificial lipid bilayer membrane systems have shown that exposure to an external electric field results in the generation of a transmembrane potential, and the short-lived steady-state current across the membrane induces a heightened permeability to hydrophilic molecules. Similar assessments to measure the changes in free energy across synthetic membrane analogues substantiate this finding (Benz, 1988; Drees *et al*, 2003). Aside from an irreversible permeabilization of the cell membrane, the application of electric fields have been shown to cause cell death by directly oxidizing cellular constituents including intracellular coenzyme A without overt membrane rupture (Matsunaga *et al*., 1992). It appears that cell size and shape are the primary determinants of the PEF inactivation kinetics, and yeasts cells display the greatest susceptibility to the electrical field effects when compared to vegetative bacteria. Bacterial spores and mould ascospores displayed the most resistance to the PEF effect (Wouters *et al*., 2001; Drees *et al*., 2003).

Current research suggests that antimicrobial agents in combination with an electric current act synergistically to inactivate bacteria (Drees *et al*, 2003; Kimbrough *et al*., 2006).

3.4.4 Electro-Chemically Activated (ECA) water solutions

ECA technology is a novel refinement of established electrolytic processes for the electroactivation of aqueous solutions. This patented, unipolar electro-activation technology generates two separate and distinct solutions, generically termed Anolyte and Catholyte which correspond to their derivative electrode chambers (Prilutsky and Bakhir, 1997; Bakhir, 1999; Tomolov, 2002). Through the ECA process, aqueous

solutions have been described to acquire unique and anomalous reactive capabilities and distinctive attributes which are substantively independent of any chemical reagents that may be present (Buck *et al.*, 2002).

Conventional electrolysis refers to the modification of the solute molecules in a water solution for the production of specific chemical reagents whose quantity and quality can be predicted by the design of the system. In contrast, instead of generating chemical entities, the ECA process refers to the manipulation of the solvent water medium, whereby it acquires unique and deviant properties, the magnitude of which significantly exceeds strictly conventional physical and chemical transformations alone. In the process of electrolytic decomposition of water, particles or compounds are formed which cannot exist outside of the ECA solutions (Prilutsky and Bakhir, 1997; Bakhir, 1999).

The mixed oxidant composition of ECA solutions is reported to be a non-toxic antimicrobial agent against which bacteria cannot develop an adaptive response (Bakhir *et al*., 2003[b]). The superior antimicrobial efficacy of mixed oxidant biocides has alternatively been demonstrated by mechanically admixing different types of oxidant species. Enhancement to the efficiency of bacterial inactivation of up to 52% has been demonstrated and has been ascribed to the synergistic biocidal effects of combinations of Cl_2, O_3 and ClO_2 (Son *et al*., 2005).

Other equivalent agents including flame (heat), sunlight (UV), and an electrical discharge and all serve to produce compounds that are either metastable or which induce a state of metastability in the immediate microenvironment of the bacterium. However the use of these essentially physical agents to induce metastability coincides with a variety of adverse consequences that limit the full extent of their widespread application (Bakhir *et al*., 2003[b]). The basic physicochemical distinction of the ECA solution that differentiates it from formulated aqueous chemical solutions, is the persistent presence of an array of compounds which would normally be eliminated within a few minutes under conditions of conventional chemistry i.e. ozone, hypochlorous acid and chlorine dioxide (Bakhir *et al*,. 2003[c]).

Without the maintenance of the activated state, the ECA solutions revert to the original energy status of the benign feed solution and the anomalous attributes of the activated solutions such as a substantially elevated oxidation-reduction potential, heightened conductivity and altered surface tension similarly decay through a process of relaxation to their pre-activation status (Tomolov, 2002). Relaxation is an irreversible thermodynamic process, and as such should dissipate the evolved energy as heat. However, electrochemical relaxation generates only a limited temperature change and the majority of energy transfer is coupled to the thermodynamic disequilibrium as evidenced by the exaggerated REDOX shifts (Bakhir, 1999).

The basic elemental analysis of the ECA solutions relative to that of brine or a non-halide salt based feed solution confirms the charge based partitioning of the monovalent sodium cation in the cathodal chamber, as well as describes the anomalous shifts in the carbonate and hydroxyl moieties of the reducing catholyte solutions. These shifts are a reflection of the total anion-cation mass balance and describe a distinctly skewed relationship as a result of the electroactivion process (Table 2) (Claassens, 2002).

Table 2. Comparisons of the concentrations of the electrolyte constituents between different non-activated feed solutions and their derivative anolyte and catholyte products.

Solution type	pH	EC mS/m	Na mg/l	Cl mg/l	CO_3 mg/l	HCO_3 mg/l	OH mol
Softened Water	7.9	24	52	10.6	0	106	$8.4x10^{-8}$
NaCl + water	8.2	645	1590	2139	2	116	$3.2x10^{-6}$
NaCl Anolyte	7.1	587	1630	1885	0	127	$3.9x10^{-8}$
NaCl Catholyte	11.7	665	1610	1733	421	-326	$2.0x10^{-2}$
$NaHCO_3$ + water	9.7	384	990	29.7	585	402	$6.1x10^{-6}$
$NaHCO_3$ Anolyte	7.3	138	340	20.6	0	851	$1.2x10^{-7}$
$NaHCO_3$ Catholyte	11.8	383	640	9.6	1049	-822	$1.3x10^{-4}$

In the face of increasing microbial resistance to current biocidal and antimicrobial remedies, a critically important attribute of the ECA solutions has been the inability to

induce resistance despite widespread and extended applications. Due to the metastability of neutral anolyte, it does not accumulate in the environment and its lack of active or hazardous degradation bi-products precludes the likelihood of toxic residues and the adaptation of microflora to the same (Bakhir *et al*., 2003[a]).

Further to the description of the prerequisites of an ideal biocide detailed by Block (1991), it has been further proposed that the current requirements of an effective biocide should comprise:

1. That the biocidal agent must demonstrate the broadest spectrum of antimicrobial capacity within the shortest exposure time, and that it should possess properties which prevent the target microbes from developing any tolerance or resistance under conditions of repeat exposure.

2. The biocidal agent must be safe for non-target organisms irrespective of the duration of exposure i.e. acute or chronic. In addition, it should not produce xenobiotic degradation products which may be potential environmental pollutants.

3. The biocidal agent should be universal in its action i.e. display broad spectrum antimicrobial efficacy, co-detergency, be free of residues, compatible with all in-contact materials, cost effective and user friendly (Bakhir, *et al*., 2003[c])

Neutral anolyte generated by the recirculation of the reducing catholyte solutions through the anodal chamber is a transparent liquid, pH neutral with synergistic detergent and disinfectant properties. It has pluripotential antimicrobial efficacy, is free rinsing, residue free, and degrades to the benign status of the dilute brine solution after relaxation. Despite the heightened electrical activity and altered physico-chemical attributes of the ECA solutions, they remain non-toxic to mammalian tissue and the environment. Current studies have shown that neutral Anolyte has no mutagenic, carcinogenic, embryotoxic or immunotoxic effects (Bakhir, *et al*., 2003[a]; Panichev, 2006).

The capacity to adjust the hydraulic flow configuration of the brine solutions during electroactivation permits the customisation of the ECA solutions such that their biocidal activity becomes specifically tailored to the prevailing environmental conditions as well as the bacterial type and the degree of bioload present. By making adjustments to the pH, flow rate, mineralization and power input during generation of the ECA solutions, it is possible to produce substantive shifts in the biocidal efficacy of the products. This will primarily reflect in the change in the Free Available Chlorine concentration, but will also encompass other predictive biocidal characteristics such as REDOX potential.

Aside from the demonstrated detergency (Hennion, 2006), ECA solutions have also been reported to be a dominant biofilm removal and regrowth control intervention (Marais and Brözel, 1999; Marais, 2000; Cloete, 2002; Ayebah *et al.*, 2005; Thantsha and Cloete, 2006).

The ECA solutions do have a finite half-life of activity, and while the anolyte will retain its biocidal potential under optimal storage conditions (Len *et al.*, 2002), the antoxidant properties of the catholyte are rapidly degraded and have been reported to display a half life of less than 8 hours when exposed to ambient environmental conditions (Bakhir, 1999).

3.4.4.1 Mechanism of action

It has been reported that both the stable and metastable products of the electrochemical activation process impact directly upon the lipid membranes, intracellular structures and cytoplasmic molecular complexes (Bakhir, 1999, Diao *et al.*, 2004). Additionally it has been proposed that the oxidizing and reducing components that comprise the ECA solutions disrupt the dynamic REDOX potentials of both the peri- and intracellular microbial milieu, and thereby modifiy and overwhelm the metabolic balance and regulatory capacity of the endogenous oxidant and antioxidant systems (Prilutsky and Bakhir, 1997; Miroshnikov, 1998; Buck *et al.*, 2002; Kimbrough *et al.*, 2006).

Simplistically put, the presence of a heightened oxidant capacity in the proximity of a bacterium is proposed to scavenge electrons away from the barrier structures causing it to become destabilised and leaky and where the loss of membrane integrity ultimately results in cell death (Suslow, 2004). While untested, this hypothesis presents should be viewed as a largely speculative perspective on the proposed mechanism of action

As reported earlier (Prilutsky and Bakhir, 1997), anolytes can be generated over a wide pH range and the reactive oxidant species that are generated under different pH conditions will result in solutions with distinctly different compositions and reactivities. Aside from pH, variations in brine mineralization, activation of non-halide salt solutions, flow dynamics, reactor design and current input will all influence the composition of the final anolyte solution produced (Sampson and Muir, 2002). Thus to ascribe any definitive causal relationship between the vast array of different types of electrolytically generated anolyte solutions and the equally diverse array of bacterial cellular changes that have been reported, would not be valid at this time. While considerable advances have been made in describing the ensuing changes that follow exposure to an oxidant biocide, they largely remain a broad description of the nett inactivation effect and falls short of detailing a definitive mechanism that may link all the directed changes to a specific disruption or a singular causal event. Despite the limitations of the current technology to adequately describe the specific microbial changes associated with anolyte exposure, there does appear to be a substantial degree of overlap in the type of cellular disruption that has been reported (Bakhir, 1999; Zinkevich *et al*., 2000; Diao *et al*., 2004; Suslow, 2004; Liao *et al*., 2007).

In addition most studies detail the changes associated with exposure to undiluted anolyte solutions wherein neither the time frame nor the magnitude for the change has been quantified or qualified. In such cases, the reports detail only gross cellular destruction without contributing any substantial refinement to the specific causes. Progressive refinements and combinations of different technologies have permitted the greater chronological characterisation of biocidal effect and Liao et al. (2007) were able to demonstrate the progressive changes associated with initial outer wall damage and the subsequent leakage of ß-galactosidase that follows damage to the ECA exposed bacterial cytoplasmic membrane.

Aside from the largely superficial mechanistic categorisation of exogenous oxidising biocides described previously, it is also critical to recognise the substantive role played by the initiation of the cascade of secondary endogenous oxidative reactions and the consequential autocidal disruption to critical cellular structures and physiological processes that would follow. Given the highly complex nature of the inactivation process, it is proposed that the most plausible explanation would rest with tracing the sequence of reported changes that arise when a microbe encounters an oxidant compound or agent with biocidal potentiality.

In order to properly qualify the type of anolyte solution to be evaluated, it is necessary to prescribe the ECA production parameters in terms of reactor type, concentration and type of salt solution, flow rate as well as the energetics of the electroactivation process. While the inciting biocidal agent has largely been referred to as being a tangible or quantifiable entity, it must also be acknowledged that the catalyst for microbial inactivation may also be ascribed to a deviation in the electronic or energetic milieu of the bacterium that would exceed the intrinsic capacity of the same to adapt to or accommodate the variance so as to ensure its continued viability and vitality.

As a point of departure, it is the cell barrier that first encounters the effects of the biocidal agent. It has been reported that the electronic equilibrium of cellular membranes is substantially determined by the ratio of saturated to unsaturated fatty acids that comprise its structure (Thomas and Aust, 1986; Bakhir 1999). Every phospholipid in every membrane of every cell contains an unsaturated fatty acid residue and the high concentration of polyunsaturated fatty acids in phospholipids makes them prime targets for reacting with oxidizing agents (Fridovich, 1979; Marnett, 2000; Spickett *et al*., 2000). As a consequence, all the chemical ingredients as well as the transitional metal catalysts required for free-radical oxidation are ubiquitous in the living cell (Dormandy, 1978).

However it is important to note that not all oxidant agents display equivalent disruptive properties, and it has been reported that superoxide and hydrogen peroxide do not peroxidize membrane lipids or degrade DNA (Halliwell *et al*.,1985). Both superoxide and hydrogen peroxide are poorly reactive in the aqueous solution, and it

has been proposed that their definitive biocidal capacity may be related to the formation of more reactive derivative species of radicals. Similarly it has been detailed that H_2O_2 can readily cross cell membranes while the superoxide radical requires a specific anion channel for cross membrane transport (Halliwell *et al*.,1985).

The carbon diene bonds of unsaturated fatty acids have been shown to possess strong electron-donor properties, and these foci of reducing capacity are localised due to the fixed nature of the C=C diene bonds on the membrane surface (Bakhir, 1999; Spickett *et al*., 2000). It is these unsaturated fatty acids with which hypochlorous and hypobromous acids will readily undergo an electrophilic addition reaction to form the substantially more reactive chlorohydrins and bromohydrins respectively (Carr *et al*,. 1998, Spickett *et al*., 2000).

Aside from the susceptibility of unsaturated fatty acids to selective oxidants, it has been shown that the exposure of the membrane associated lipids to hypochlorous acid will result in the decomposition of lipid hydroperoxides into peroxyl radicals which are a potential source of the highly reactive singlet oxygen $^1O_2$ (Halliwell *et al*,. 1985; Marnett, 2000; Miyamoto *et al*., 2007).

Lipid hydroperoxides are the initial but short lived bi-products of unsaturated fatty acid oxidation. When considering the reaction of lipid hydroperoxides and hypochlorous acid as a REDOX reaction, it is expected that hypochlorous acid would be reduced to Cl^-, and that the hydroperoxides would be oxidised to peroxy radicals (Spickett, *et al*., 2000). The lipid hydroperoxides either become reduced to non-reactive fatty acid alcohols by the limited reserves of glutathione peroxidases, or they will react with metals to produce a variety of products which are themselves highly reactive (e.g. epoxides, aldehydes, etc.). Of these, malondialdehyde and 4-hydroxynonenal are the predominant metabolites, and while their formation describes a lag phase, both are recognised to display significantly additive toxic attributes (Marnett, 2000; Spickett *et al*., 2000).

This proclivity of polyunsaturated fatty acid molecules to become oxidized has resulted in the evolution of an extensive system of polycyclic antioxidant compounds and enzymes to safeguard against autocatalytic membrane oxidation (Marnett, 2000).

The critical role of these protective mechanisms has been confirmed and while recognising that there is a direct correlation between SOD content and oxygen tolerance, the suppression of the MnSOD enzyme of a soluble extract of *Streptococcus faecalis,* resulted in a 17% conversion of absorbed oxygen directly into the superoxide radical (Fridovich, 1979).

It is estimated that 60 molecules of linoleic acid (the most common polyunsaturated fatty acid in cells) are consumed per oxidant molecule that reacts with the phospholipid bilayer (Marnett, 2000). In the face of a self perpetuating autocatalytic cascade, and given that one anti-oxidant molecule can only scavenge one free radical, it is critical that all vital cells must posses a constantly self-generating antioxidant potential (Dormandy, 1978; Guentzel *et al*., 2008). A recent study by Liao *et al*. (2007) proposed that oxidant stress disrupts the REDOX state of the Glutathione antioxidant system with consequential damage to the metabolic pathways and cell necrosis. Thus it becomes incontrovertible that a surplus of initial exogenous and incremental secondary endogenous oxidant activity would readily overwhelm the intrinsic antioxidant capacity of the cell and thus render it physiologically susceptible to irreversible disruption. However despite the substantial body of data to support the biochemical changes described, it has been reported that the modification of cell membrane proteins occurs at substantially lower doses of hypochlorous acid than would be required for chlorohydrin formation, and that the threshold for cellular lysis describes a defined concentration dependent effect (Spickett *et al*., 2000).

The overt changes to the ORP gradient across the cell wall and membrane directly impacts both the passive, but more so the active transport of substances into the cell due to the disruption of the electro-osmotic gradient across the membrane. Coupled to this, the structurally altered and numerically reduced clusters of water molecules display a heightened diffusion potential into the cell and aside from causing a direct shift in the osmotic pressure, the surplus water will also readily catalyse a number of biochemical reactions that would normally be dependent on the physiologically limited presence of water molecules. The diverse matrix of chemical reactions that occur in association with a biocidal incident have largely clouded the qualified apportionment of a direct causal relationship between the myriad of active chemical compounds described and the ultimate loss of cellular viability. In addition,

conventional electrolytic processes employing the catalysis of NaCl have presumptively accorded the nett biocidal effect to the action of the chlorine species so generated. Despite the concurrent presence of a diverse range of reactive oxygen species (ROS), attributions of biocidal activity have largely focussed on descriptions of the potentiation of the recognised antibacterial capacity of the chloride ions by these ROS.

Evidence would suggest that it is a broad based synergy of the various reactive oxidant species that are responsible for the bulk molecular disruption that have been reported, and that the magnitude of the cytoplasmic damage that arises is largely due to the activity of either autooxidative or localised endogenous metabolites whose actions are secondary to the primary oxidative insult. At the cytoplasmic level it has been reported that oxygen radicals attack all cellular macromolecules and not just DNA. It has been shown that the cytotoxicity of superoxide and hydrogen peroxide is directly linked to the availability and location of metalo-catalysts of HO· production, and it has been demonstrated that the killing of *Staphylococcus aureus* by hydrogen peroxide becomes substantially more effective if the internal iron content of the cell is augmented (Haliwell, 1985). Estimates suggest that the DNA molecules may be the least significant target from a standpoint of quantitative damge. Notwithstanding, it has been reported that the levels of oxidative DNA damage arising from endogenous sources substantially exceeds that of the levels of lesions directly induced by exposure to the exogenous compounds alone (Marnett, 2000).

The energetic basis of the direct oxidant damage to biomolecules as well as the linear and thus gravimetric relationship elaborated by the neutralisation to the oxidant effect by an antioxidant agent has been evaluated. When fixed quantities of an electrolytically derived reducing solution was titrated into a suspension of oxidant damaged bacterial cells, the antioxidant water was demonstrated to scavenge hydrogen peroxide, superoxide, singlet oxygen as well as the hydroxyl radicals and was shown to substantially suppresses the single strand breakage of DNA and other damage typically associated with reactive oxygen species (Shirahata *et al.*, 1997). However, while the reduced water significantly reduced the single strand breakage of DNA in a dose dependent manner, the diminished inactivation of the hydrogen peroxide relative to that of the superoxide radical would suggest that the ultimate

damage to DNA is predominantly attributed to hydrogen peroxide and its more reactive metabolites (Shirahata *et al*., 1997). This assertion is supported by the rapid peroxide induced exhaustion of endogenous catalyse enzyme reserves previously reported by Block (1991).

While it is likely that the hydroxyl radical (HO·) plays the most significant role in the endogenous oxidation of DNA, the reactivity of HO· is so great that it does not diffuse more than one or two molecular diameters before reacting with a susceptible cellular component (Marnett, 2000).This is confirmed by the finding that the reactive time of the hydroxyl radical is of a very short duration i.e. <1µs, and hence it will only react in close proximity to the site where it is formed (Hill, 1979). Thus in order for HO· to oxidize the components of a DNA strand, it must be generated immediately adjacent to the nucleic acid molecule. Given its highly labile reactivity, it is thus likely that H_2O_2 serves as a readily diffusible latent form of HO· which reacts with a metal ion in the vicinity of a DNA molecule to generate the destructive oxidant radical. An equivalent but little known reactive endogenous ROS metabolite is peroxynitrite (ONO_2^-) and its ability to readily diffuse within cells via anion transporters may serve to explain the spatially disparate secondary endogenous oxidation of DNA (Marnett, 2000). In addition to the generation of cytotoxic membrane associated lipid hydroperoxides, other biological hydroperoxides derived from cytoplasmic proteins and nucleic acids will also participate in reactions that lead to secondary ROS and specifically singlet oxygen generation (Miyamoto et al., 2007).

While Zinkevich *et al*., (2000) reported that a 5 minute exposure to the ECA anolyte solutions resulted in the total destruction of both chromosomal and plasmid DNA of vegetative bacteria, a separate study on the treatment of *Bacillus subtilis* spores with the same anolyte solutions did not result in any significant DNA damage (Loshon *et al.,* 2001). Instead it was proposed that the sporicidal action of the anolyte solution was due to the oxidative modification of the inner membrane of the spore. While all anolyte damaged spores were reported to undergo the early stages of germination which included dipicolinic acid release and cortex degradation, all germinated spores displayed an increase in cell wall permeability (Loshon *et al.,* 2001).

In an attempt to quantify the extent to which the electronic 'activation contribution' augments the reagent based biocidal capacity of ECA solutions, the relative biocidal efficacy of a hypochlorous acid solution of equivalent strength generated from the acidification of sodium hypochlorite was contrasted against that of a hypochlorous acid solution generated from an ECA process (Shimizu and Sugawara, 1996). It was demonstrated that sodium hypochlorite at a concentration of 100mg/L (9 mg/L Cl), had no virucidal effect against polio virus, but that the addition of hydrochloric acid to the same solution did increase the virucidal effect. Chemically derived hypochlorous acid with a free chlorine concentration of less than 4.5mg/L displayed no microbicidal effects against *E. faecalis*, while the same challenge titre was eliminated with an electrolyzed oxidizing anolyte solution that had a free chlorine concentration of less than 2.1mg/L. The same superior virucidal effect was demonstrated with the equivalent virus titres that were previously resilient to exposure to the chemically derived hypochlorous acid exposure (Shimizu and Sugawara, 1996).

In a similar study to test the inactivation efficacy of a mixed oxidant solution derived from a plate based electrolysis device relative to that of an equivalent concentration of a chlorine based compound, the mixed oxidant ECA solution was able to achieve a >3 $\log_{10}$ inactivation against both *Cryptosporidium parvum* oocysts and *Clostridium perfringens* spores within 4 hours, while the equivalent chlorine based solution had no effect against *C. parvum* and only achieved a 1.4 $\log_{10}$ inactivation of *Cl. perfringens* over the same time period (Venczel *et al*., 1997).

The Minimum Microbicidal Concentration (MMC) of ECA anolyte against Herpes simplex type 1, Polio virus and *Enterococcus faecalis* was demonstrated to be less than that of hypochlorous acid generated from equivalent chemical analogs. The enhanced virucidal and bactericidal effects of the ECA anolyte was proposed to be due to the synergism with the other reactive oxidant species that are present in the anolyte (Shimizu and Sugawara, 1996). While a heightened biocidal effect has been demonstrated with electrolytically generated hypochlorous acid relative to that of the parent chemical compound, recent reports have indicated that the enhanced microbial inactivation cannot be fully explained exclusively on the basis of the action of the electrochemically generated chlorine based compounds alone (Kimbrough *et al.* 2006). In a recent study, electrochemically activated anolytes were produced by a

plate reactor using chlorine free, phosphate based buffers at a neutral pH. Through selective neutralisation of the hydroperoxy-radicals generated during electrolysis using *tert*-butyl alcohol and sodium thiosulphate, a causally associated attenuation of ROS induced microbial activation was described (Jeong *et al*., 2006). This finding is supported by the relatively enhanced biocidal capability of the ROS reagents of electrochemical activation when compared against the products of a simulated Fenton reaction (Diao *et al.*, 2004).

Further independence from an exclusively halide reagent based biocidal effect has been reported wherein the biocidal reactivity of carbonate based radicals were shown to display substantially extended longevity relative to that of hydroxyl radicals, despite the former being substantially less reactive (Hill, 1979). The antimicrobial efficacy of chlorine free sodium bicarbonate derived anolytes has also been described (Malherbe and Cloete, 2001; Kirkpatrick, 2005; Thanthsa and Cloete, 2006).

Aside from the definitive role of the reactive oxygen species generated during the non-halide electrolysis, the suspensions of *E.coli* exposed to these electrolytic solutions displayed a progressive inactivation which correlated to an increasing electronic anodal potential and current density (Diao *et al*., 200; Jeong *et al.*, 2006). This finding coincides with the enhanced bacterial and viral inactivation previously described using variations with applications of direct electric current (Drees *et al.*, 2003, Kimbrough *et al*., 2006).

Len *et al*., (2000) reporting on spectrophotometric studies of electrolyzed oxidizing (EO) water, have suggested that the primary pH dependent antimicrobial effect was directly related to hypochlorous acid concentration, and that the magnitude of inactivation was correlated to the amperage applied across the electrodes and hence the incremental stoichiometric quantity of chloroxy based compounds so generated. Thus while the specific reasons for inactivation of microbial cells by ECA solutions remains uncertain, evidence suggests that that it involves the dual actions of hypochlorous acid as well as that of an enhanced REDOX potential (Liao *et al.*, 2007). This proposal is supported by the evidence that bacterial inactivation by hypochlorous acid was due to a combination of the oxidation of cell surface sulfhydryl compounds, the inactivation of respiratory enzymes, the inhibition of ATP

generation, and the the retardation of active transport (Park *et al*., 2002, Liao *et al*., 2007).However it has recently been speculated that it is the REDOX that might be the primary factor that results in microbial inactivation (Kim *et al*., 2000; Park *et al*., 2004), as it has been demonstrated that the elevated REDOX was responsible for the substantially greater microbial inactivation relative to a chlorinated water solutions of equivalent concentration (Liao *et al*., 2007). It is now suggested that the modification of the metabolic fluxes and the disruption to ATP production is largely due to the ORP induced changes to the patterns of electron flow within the bacterial cells (Park *et al*., 2002).

3.5 Conclusions

The delicate balance of molecular and electronic structure that confers life to a micro-organism is perpetually exposed to adverse conditions that may impact upon its viability. A variety of protective mechanisms have developed in response to these stressors and many have become encoded in the genetic template of the microorganism.

Exposure to noxious chemical compounds results in a diverse range of cellular disruptions. While not all changes to cellular structure and function are life threatening, prolonged sub-lethal exposure to a physical effect or chemical compound affords the individual cells within the exposed population with the chance to reinforce the tolerance mechanisms which will lead to a stable resistance to the offending agent.

While the composition of electrolytically generated solutions continues to undergo rigorous analysis, scant attention has been applied to the the role of the REDOX potential in describing it role as a composite and adjunct antibacterial agent. In conjunction with the specific roles played by the chemical constituents of the ECA electrolytes, it is proposed that the enhanced antimicrobial efficacy of the ECA solutions is substantially due to the disruptive effect that the exaggerated ORP state exerts on the bacterial microenvironment.

It is thus proposed that microbial exposure to ECA solutions initiates an autocidal cascade of events that starts with disruption of the electronic microenvironment of the

outer membrane, leading to an irreversible disturbance to the electron-motive force responsible for the maintenance of the chemiosmotic balance across the barrier membranes. This in turn disrupts the oxidative phosphorylation pathways which results in an imbalance to the concentrations of toxic metabolites derived from oxygen metabolism. Irreversible microbial inactivation follows as a direct consequence of the adverse changes that the ensuing Reactive Oxygen Species will effect to the DNA, proteins (enzymes) and lipids of the cytosol. This coincides with the simultaneous and autocatalytic lipid peroxidation of the membranes and the general compromisation of physical barrier integrity and physiologic functionality.

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Chapter 4

Review of general ECA solution applications

4.1 Food applications

The clear benefits of integrating the ECA technology and its derivative solutions into food production and processing industries have produced a vast array of reports where the technology has been assessed for the control of bacterial contaminants, general spoilage and its impact on the shelf life of perishable produce. Owing to its natural ingredients, it has widespread accreditation as a food grade disinfectant and in some instance it is recognised as a core ingredient in the food and beverage manufacture sectors.

Al-Haq *et al*. (2005) have compiled the results of an extensive survey of the available literature where the ECA technology has been employed in the food industry, and detail the diverse range of electrolytic solution types assessed as well as the plethora of descriptive acronyms under which the technology has been described to date. This survey also details an equally significant number of different types of ECA devices, each with variable electrode design reactor configuration, and consequently a distinctive type of end product solution.

The majority of reports describe studies using electrolysed solutions generated from a plate or non-membrane based reactor system which lacks the capacity for pH modulation (Fig 1). In all cases, the various authors report on applications of acidic electrolyzed solutions generated at a pH < 3, which in accordance with conventional electrochemistry will primarily comprise of volatile dissolved chlorine gas in solution (White, 1992).

It is not surprising that the primary mechanism of action of these solutions has been ascribed to being a function of a combination of low pH and high available chlorine concentration (AAC), and that some Japanese authors reporting on the use of local technology, refute the option that ORP may play a role in microbial inactivation (Al-Haq *et al*., 2005).

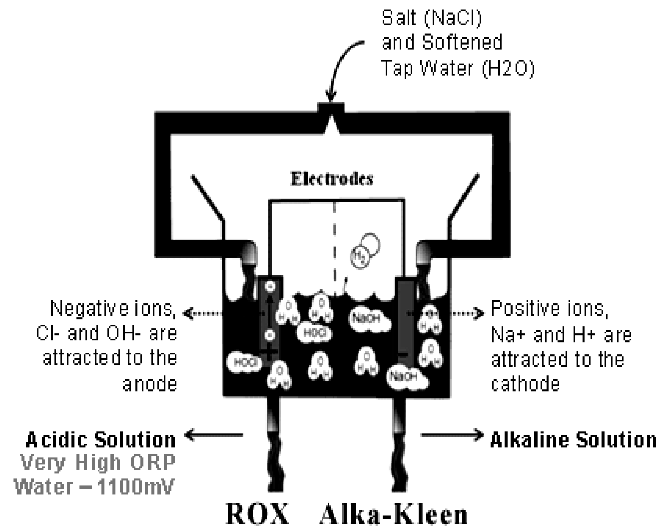

Figure 1. Schematic of the Japanese electrolytic system (Hoshizaki Electric Inc, Japan) used for the production of Acidic electrolyzed water (AEW). (www.hoshizaki.com)

The ROX electrolyser systems produced by Hoshizaki Electric are reported to activate dilute brine solutions (0.05-0.1% NaCl) to produce oxidant solutions with a pH of 2.5-2.8, a Free Available Chlorine (FAC) concentration of 32-35 mg/liter and a REDOX potential of +1100mV (Al-Haq *et al.*, 2005).

While effective in the control of superficial spoilage and pathogenic bacteria, a number of reports confirm the adverse consequences of the low pH which includes equipment corrosion, operator discomfort due to chlorine vapours, solution instability, phytoxicity, taint and bleaching of the colour of treated perishable vegetables (Al-Haq *et al.*, 2005; Guentzel *et al.*, 2007; Fujiwara *et al.*, 2009). Despite these limitations, the AEW solutions have been assessed for the control of spoilage and pathogenic bacteria and for shelf life extension in a diverse array of food products ranging from whole and processed fruit and vegetables, grains and allied products, poultry, sea food, and red meats (Park *et al.*, 2001; Al-Haq *et al.*, 2005; Fabrizio and Cutter, 2005; Kim *et al.*, 2005; Huang *et al.*, 2006). The substantial effect of ECA solutions against biofilms has previously been described by Thantsha and Cloete (2006).

In converse, relatively few authors have conducted studies using the neutral anolyte produced with the proprietary coaxial Flow Electrochemcial Module (FEM) reactor system as patented and commercialised by Bakhir *et al.*, (1995, 1996, 1998, 1999).

Guentzel *et al*. (2008), reported on the use of the neutral anolyte generated from a FEM 3 based reactor, and confirm that the near neutral (pH = 6.5) electrolysed oxidising (EO) water was comprised of hypochlorous acid (~95%), hypochlorite anion (~5%) with trace amounts of Chlorine. They suggest that the high ORP of the solution is disruptive to the integrity of the outer cell barrier, which results in an increased permeability to the HOCl molecule and the subsequent oxidation of cellular reactions and disruption of respiratory pathways.

A 10 minute dip treatment of harvested spinach with a challenge innoculum of *E.coli, Salmonella typhimurium, Listeria moncytogenes, S. aureus* and *Enterococcus faecalis* using neutral oxidant ECA derived solutions, resulted in 5 log_{10} reduction of all strains within the same time period (Guentzel *et al*., 2008). A similar study using AEW on lettuce leaves resulted in a 2.6 log_{10} reduction after 3 minutes exposure, and the authors confirmed that the antimicrobial efficacy of the AEW was insignificantly different from that of an acidified chlorinated dip wash (Park *et al*., 2001).

The use of the neutral electrochemically activated solution for the decontamination of poultry carcases artificially challenged with *Salmonella typhimurium* resulted in a 1.39 log_{10} reduction when sprayed for 17 seconds. A hypochlorite spray at an equivalent FAC of 50 mg/L resulted in a 0.86 log_{10} reduction (Yang *et al*., 1999).

When chicken skin challenged with pure cultures of *Campylobacter jejuni* were exposed to a variety of dilutions of EAW solutions, it was reported that solutions of high residual chlorine concentrations i.e. >50mg/L displayed a comparable antimicrobial effect relative to that of a chlorine solution of the same chemical concentration. However when the same challenge dose of bacteria was exposed to an EAW dilution of lesser strength and hence reduced residual chlorine concentration i.e. < 25mg/L, the comparable chlorine solution was unable to effect an equivalent reduction in viable count. This confirms the intrinsic capacity of the EAW solutions to exert a greater antibacterial effect relative to standard chlorine based solutions, and substantiates the assertion that the biocidal attributes of the EAW technology exceeds that of purely chemically based formulations (Shimizu and Hurusawa, 1992, Kim *et al*., 2000; Park *et al*., 2002).

It has been reported that exposure of poultry carcases to a 28mg/L hypochlorous acid solution derived from a mixture of $Ca(OCl_2)$ and HCl, resulted in the development of tolerance by a *Salmonella* isolate within a poultry abattoir (Mokgatla *et al*., 2002). The study details substantially reduced levels of superoxide dismutase and increased levels of catalase enzymes in the HOCl tolerant microbial strain relative to that of a wholly susceptible *Salmonella* strain, and substantiates the findings of an earlier study suggesting that hypochlorous acid exposure can induce similar adaptive responses to that of hydrogen peroxide stress in *E.coli* (Dukan and Touati, 1996). It would be of critical interest to assess the response of the tolerant strain when exposed to a neutral oxidant solution at an equivalent FAC.

The heightened public awareness of the importance of pathogenic diseases associated with Ready-to-Eat (RTE) food products has resulted in a number of studies on the antibacterial efficacy of Electrolysed oxidising water against amongst others *Escherichia coli* 0157:H7, *Salmonella enteriditis* and especially *Listeria monocytgenes* (Venkitanarayanan *et al*., 1999[a,b]; Park *et al*., 2004; Fabrizio and Cutter, 2005).

While the use of acidic electrolysed water (AEW) was shown to be effective in reducing all of the abovementioned pathogens in suspension (Venkitanarayanan *et al*., 1999), it was acknowledged that the application of the AEW solution to the RTE meat products (i.e frankfurters and ham) was ineffective in meeting the current USDA-FSIS requirements of > 2 $\log_{10}$ reduction in *L. monocytogenes* during further meat product processing and packaging (Fabrizio and Cutter, 2005).

Muriana (2005) studied the antimicrobial effects of neutral electrolyzed water (EW) produced by a FEM based reactor system for the decontamination of the processing equipment used with RTE meats contaminated with strongly adherent strains of *Listeria monocytogenes.* Both clean and soiled delicatessen slicing blades contaminated with *L. monocytogenes* were treated with a fine mist spray of sterile distilled water, and a 1:10 and an undiluted EW solution for 15 seconds.

While the sterile distilled water spray was shown to rinse off some of the loosely adherent innoculum, the spray applications of the 1:10 dilution and undiluted oxidant

solutions on the clean blades resulted in a 3.6 $\log_{10}$ and >5.66 $\log_{10}$ reduction of *L. monocytogenes* respectively (Fig 2).

When the antimicrobial effect on the soiled slicing blades was assessed, the 1:10 dilution and undiluted EW solutions displayed a significantly reduced efficacy i.e. 0.64-$\log_{10}$ and 3.34-$\log_{10}$ respectively (Fig 2) (Muriana, 2005).

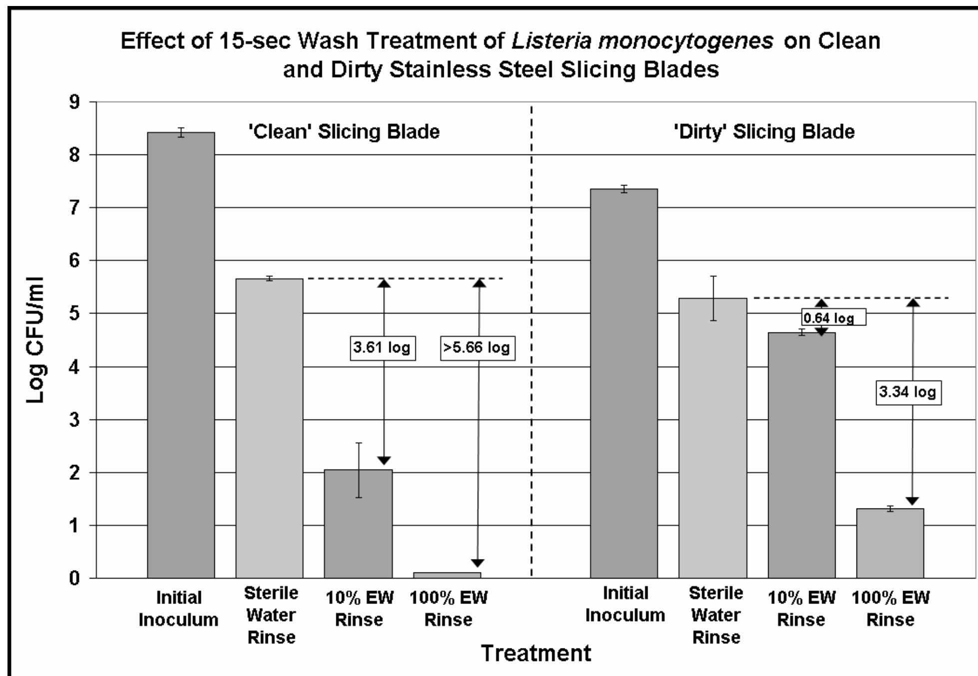

Figure 2. The effect of a 15 second spray treatment of Electrolysed Water (EW) on *Listeria monocytogenes* on clean and dirty stainless steel cutting blades (Muriana, 2005).

An unpublished report complementary to the Muriana study indicated that a 3 minute spray or immersion in an undiluted neutral EAW solution derived from an identical reactor system, resulted in a consistently greater than 2 $\log_{10}$ reduction on frankfurter surfaces contaminated with *L. monocytogenes* (Fig 3) (Kirkpatrick and Bagnall – unpublished data, 2005).

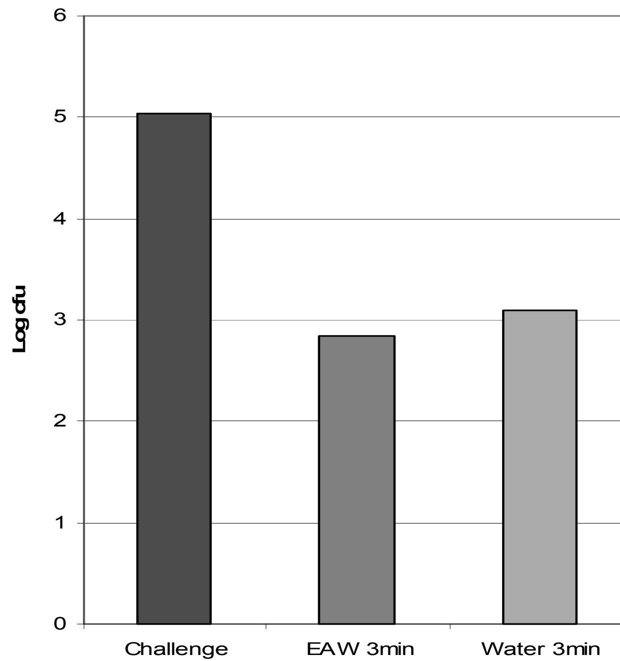

Figure 3. *Listeria monocytogenes* (indicated as log CFU) recovered from frankfurter surfaces after a spray treatment with undiluted EAW and sterile distilled water.

4.2 Oxidation effects

Aside from the widely reported antimicrobial action of the electrochemically activated oxidant solutions, conventional chlorine based formulations have been extensively used for the oxidative manipulation and toxin neutralisation of a variety of chemical compounds. Included here, are the microbially derived toxins which comprise of fungal mycotoxins and bacterial entero- and exotoxins, the inorganic pesticides, herbicides and heavy metals (Prilutsky and Bakhir, 1997).

The use of acidic anolyte (pH 2.5-2.8) to oxidise chemically pure fractions of aflatoxin and Staphylococcal Enterotoxin-A have been reported (Suzuki *et al*., 2002a; Suzuki *et al*., 2002b), and in both instances the inactivation kinetics were suggested to be strongly dependent on the dose of both the hypochlorous acid as well as the hydroxyl radicals present in the acidic oxidant solutions.

In both cases, it was confirmed that the reducing and negatively charged catholyte solution was ineffective in the decomposition of the microbal toxins (Suzuki *et al*., 2002[a]; Suzuki *et al*., 2002[b]). This finding is at odds with the results of the assessments

conducted on the use of the reducing solutions to decontaminate mycotoxins on whole maize kernels, and which will be reported later.

The impact on pesticide and fungicide residues by the oxidant ECA solution has also been evaluated. The oxidant ECA solution was diluted using a 10 fold dilution series and the potential for non-ECA based hydrolysis or unrelated chemical breakdown was excluded by including two untreated control solutions which comprised of both the tap water used as the diluent solution of the oxidant ECA dilution series as well the diluted, non-activated brine solution that was used as the electrolysis feed solution prior to electro-activation. The physicochemical parameters of each solution type were measured before the experimental exposure (Table 1).

Table 1. Physico-chemical properties of the tap water, non-activated salt solution and the diluted oxidant ECA solutions used in the pesticide exposure.

Solution type	ORP (mV)	pH	EC (mS/cm)	FAO (ppm)
Tap water control	280	8.2	0.21	-
2.5gm/lit salt solution	290	7.7	5.22	-
1% Anolyte solution	436	7.5	0.35	≤ 5
10% Anolyte solution	803	7.2	1.34	20-25
100% Anolyte solution	940	6.5	5.45	≤ 200

Legend: ORP – Oxidation-Reduction Potential (mV-milliVolts), EC- Electrical Conductivity (mS/cm – milliSiemens per centimete), FAO – Free Available Oxidant concentration (mg/litre, ppm-parts per million).

The experiment was performed to assess the measure of breakdown of a variety of pesticide and fungicide active ingredients (AI's) after they were exposured to a tap water control, an inactivated brine solution, and a variety of diluted ECA oxidant solutions. A 1 ml aliquot of a cocktail of each of the active ingredients was added to 100ml of the different test and control solution samples. The test samples were agitated with a mechanical stirrer for 5 minutes at ambient temperature and then extracted with an organic solvent and analysed by either gas or liquid chromatography (Table 2).

Table 2. Schedule of pesticide compounds exposed to Anolyte solutions at different dilutions and the percentage breakdown after exposure for 5 minutes.

Active Ingredient	Pesticide Category	1% Anolyte	10% Anolyte	100% Anolyte
		Percentage breakdown		
Malathion	Organophosphorus insecticide	0.0	100	100
Chlorpyrifos	Organophosphorus insecticide	0 0	100	100
Cyprodinil	Anilino-pyrimidine fungicide	0.0	96.3	100
Kresoxim-methyl	Strobilurin fungicide	0.0	0.0	100
Bupirimate	Pyrimidine Fungicide	0 0	52.0	100
Azinphos-methyl	Organophosphorus insecticide	0 0	100	100
Benomyl	Benzimadazole fungicide	8.0	45	100
Aldicarb	Carbamate insecticide	49.5	100	100
Aldicarb sulfoxide	Carbamate insecticide	15 8	100	100
Methomyl	Carbamate insecticide	36.6	100	100

The study showed that the exposure of both pesticide and fungicide compounds to the oxidant ECA solutions resulted in the oxidative breakdown of the active ingredient of all formulations. The organophosphorus and carbamate group of pesticides and the benzimidazole, anilino-pyrimidine, strobilurin, pyrimidine and benzimidazole based fungicides were all oxidised during exposure to the Oxidant ECA solutions (SABS, 2007).

4.3 Reducing effects

The use of the ECA solutions to reduce levels of superificial mycotoxins on grains was evaluated as an adjunct intervention to the microbial decontamination of raw grain products prior to milling. While the oxidant solution was effective in reducing the superficial vegetative fungal contaminants normally associated with suboptimal storage i.e. *Aspergillus* spp. and *Penecillium* spp., it was shown to be ineffective

against the field strains of *Diploidia maydis* and *Fusarium* spp. which specifically colonise the endosperm during cultivation (Marais, 1998).

Maize kernels with known levels of mixed mycotoxin contamination were exposed to both the oxidant and reducing solutions under controlled laboratory conditions. Given the short half-life of the reducing anti-oxidant catholyte solution, the assessments were conducted within 2 hours of solution production. In contrast to an earlier report (Suzuki *et al*., 2002b), it was found that the oxidant ECA solution had no inactivation effect on the levels of either aflatoxin or fumonisins, while a 10 minute exposure to the undiluted reducing ECA solution was able to effect a reduction of up to 99% and 75% in both aflatoxin and fumonisin concentrations respectively (SAGL, 2001; 2004).

4.4 Agricultural Applications

Published as well as commissioned studies to assess the antimicrobial efficacy of the electroactivated oxidant solution against economically significant fungal contaminants present in the irrigation water of high risk greenhouse and hydroponic crops detailed significant inactivation efficacy under *in-vitro* conditions (Buck *et al.*, 2002; Mueller *et al*., 2003).

The halogenated oxidant ECA solution was assessed over a range of concentrations and exposure periods as an antimicrobial agent for the inactivation of the microconidia of *Fusarium oxysporum cubenese* (*Foc*), a fungal pathogen of global significance responsible for Fusarium wilt or Panama disease in banana plantations. As part of the same study, the same oxidant solution was evaluated for its antimicrobial efficacy against *Fusarium circinatum*, an economically important fungal pathogen responsible for pitch canker and root rot in pine seedling nurseries.

It was found that the oxidant ECA solution applied at a 1:100 dilution rate, with a 10 minute exposure was effective in eliminating both *Foc* and *F. circinatum* at initial challenge levels of 10^4 mocroconidial spores per ml (Groenewald *et al*., 2002).

A similar study was conducted to evaluate the antimicrobial efficacy of the oxidant ECA solution against common fungal and bacterial pathogens present in intensive hydroponic systems. The oxidant ECA solution was tested over a range of concentrations and exposure periods against water borne *Pythium* zoospores, *Fusarium* condidia and the gram negative bacterium *Ralstonia*, and in all cases a 100% kill was recorded after a 10 minute exposure at a 1:10 concentration (Bagnall, 2007). However, despite favourable antimicrobial efficacy results, the *in-vivo* exposure of cucumber seedlings to the mixed oxidant solution at concentrations in excess of 1:100 resulted in severe phytotoxicity. A similar adverse physiological effect following a foliar spray of cucumber seedlings with acidic electrolyzed water was reported by Fujiwara et al. (2009). An equivalent adverse phytotoxic effect in butter lettuce seedlings was evidenced in a small-scale field trial, when ECA solutions were applied at inclusion rates in excess of 1:20 (Bagnall, 2007). Notwithstanding, the inclusion of the oxidant ECA solution into the recirculating irrigant water at a rate of 1:50 resulted in a 43% increase in biomass yield relative to the untreated control in a small scale hydroponic study (Labuschagne and Bagnall, 2003).

An assessment of the ability of the halogenated oxidant solution to inactivate Tobacco Mosaic Virus (TMV) indicated that the exposure of the virus suspension to a 1:200 strength oxidant solution for a 10 minute period, resulted in a 99% inactivation of the TMV and the prevention of lesions on the leaves of challenged tobacco seedlings (Fig 2) (Labuschagne, 2003).

Untreated Control ECA Oxidant (1:50) ECA oxidant (1:100)

Figure 2. Tobacco Mosaic Virus (TMV) lesions on leaves of virus-challenged tobacco seedlings.

4.5 Medical Applications

Aside from the various reports describing the use of ECA solutions as surface or equipment disinfectants in health care facilities (Kirko *et al*., 1999; Selkon *et al*., 1999; Shetty *et al*., 1999; Thantsha, 2002) a number of invasive medical interventions using the ECA solutions have also been reported (Devyatov and Pertov, 1997; Devyatov *et al*., 1999; Inoue *et al*., 1997; Nakae and Inaba, 2000; Landa-Solis *et al*., 2005).

A variety of commercial preparations are currently available (Oculus Innovative Sciences, California, USA) for the treatment of topical disease conditions and the use of the FEM based ECA solution for the treatment of wounds and chronic ulcers has recently been patented (Selkon, 2007). Based on ECA solutions produced by the second generation FEM 2 technology, Puricore have recently launched the Vashe™ Wound Therapy System. Based on the patent registered by Selkon (2007), Vashe™ is promoted as a safe, non-invasive, easy-to-use, wound management process, and has been reported to promote healing by reducing wound bioburden and promoting tissue repair (Puricore, 2007).

The biocompatibility of the ECA solutions have been assessed under the guiding principles for good laboratory practice (GLP) of the Organisation for Economic Co-operation and Development (OECD) as well as the US Food and Drug administration (US FDA). These assessments comprised evaluations of acute eye irritation, acute dermal irritation and acute oral toxicity (LD_{50}), and no adverse effects or consequences to their exposure were noted (Marais, 2002).

4.6 Veterinary Applications

A number of reports attest to the improved disease control and enhanced productivity of livestock reared under intensive production conditions as a result of treatment with ECA solutions (Spirina *et al*., 1997; Marasinskaya, 1999).

In a commissioned study in an intensive pig rearing farm in Denmark, the addition of Electrochemically Activated Water (EAW) to drinking water had a significantly

beneficial effect on the productivity of weaner piglets. In addition to the elimination of potential pathogenic bacteria from the drinking water, a continuous EAW inclusion rate of 1:10 in the drinking water resulted in a higher average daily weight gain and an improved feed conversion ratio in all treated piglets during the first two weeks post-weaning (Maribo, 2002).

A further study on the clinical safety for the invasive use of the ECA solutions reports on the administration of an electrochemically activated saline solution as a post-breeding intra-uterine instillation for the control of mating-induced endometritis in mares. The oxidant ECA solution was aspetically instilled into the uterus within 12 hours of breeding, and it was found that there was no significant difference in conception rates relative to the untreated controls (Annandale *et al*., 2008).

4.7 Disinfection Bi-Products (DBP's)

Historically, the focus of disinfection has been the optimisiation of sustained antimicrobial efficacy relative to the choice and dose of the disinfecting compound applied. However, despite the substantial benefits to community health, the widespread use of chlorine based compounds for the decontamination of drinking water also results in the accumulation of disinfection bi-products which have recently been somewhat tenuously incriminated in a variety of life threatening conditions.

Upward of 700 DBP's have been reported to arise from the disinfection of potable water using chlorine, chlorine dioxide, chloramines and ozone. While alternative strategies to chlorination have reduced Trihalomethanes (THMs), Haloacetic acids (HAAs) and Total Organic Halogen (TOX) levels, these alternatives have also resulted in several new priority DBP's being generated at higher levels when compared to conventional chlorination methods (Lou and Lin, 2008). In fact pre-ozonation with subsequent chloramination has been found to produce significant increases in trihalonitromethanes [chloropicrin] levels (Krasner *et al*., 2006).

A significant proportion of neonatal, cardiovascular and oncological conditions are now being linked to the chronic exposure to these DBP's, but in many cases the veracity of the causal relationships elaborated by epidemiological studies have been

found to be based on largely spurious and unmerited extrapolations. To date, no conclusive evidence has been advanced to demonstrate an incontrovertible link between chronic exposure to THM's in drinking water and a directly allied detrimental health effect, and most research suggesting the causality of the adverse reactions fail to produce statistically credible and repeatable evidence to justify the purported linkage. The inherent difficulty of establishing a direct relationship between a recognised carcinogen and an adverse reaction over an extended exposure period is the tenuous extrapolation from the effects of acute exposure studies as well as the inability to exclude the impact of uncontrolled variables such as lifestyle and inherent predisposition.

In an extensive review of adult leukaemia in Canada, it was found that while THM's may be 'particularly important' in the etiology of chronic myelocytic leukaemia, paradoxically it was proposed that it appeared as if the bi-products of chlorination were also able to afford a 'protective effect' against the incidence of chronic lymphocytic leukaemia. Additionally, the acknowledged bias in data selection and the inability to control interim lifestyle effects resulted in the paradoxical conclusion that non-smokers were at a much higher risk of developing acute lymphocytic leukaemia as a result of consuming chlorinated water (Kasim *et al.*, 2006).

The most common DBP's associated with chlorination comprise Trihalomethanes and haloacetic acids. The four most frequently monitored THM's comprise chloroform, bromochloromethane, dibromochloromethane and bromoform. Chloroform is one of the most widely researched THM's and it has been reported that in order to consume the equivalent of a single dose of an over-the-counter chloroform based cough remedy, an adult would have to consume 8 glasses of chlorinated water every day for an entire year (Freese and Nozaic, 2004). The ongoing assessment of DBP's is far from being exhausted and previously noxious compounds have been relegated down the toxicity scale as a result of the ongoing quantification of the toxicity of previously unknown bi-products. Given that bromine has a significantly greater reactivity relative to that of chlorine (White, 1992), a recent report has confirmed that bromonitromethanes are substantially more cyto- and genotoxic than their chlorinated analogues (Krasner *et al.*, 2006).

A further concern relating to the heightened awareness of DBP's, is the reliability and misapplication of the detection methods currently being employed to assess the disinfectant levels in treated water. Aside from the inappropriate choice of reagents (e.g. DPD, Indigo, Acid chrome violet K) to assess specific disinfectant concentrations, the effects of mixed oxidant species, reaction times, masking agents and ionic strength have been shown to significantly impair the integrity of the analytical assay (Gordon *et al*., 2002).

Notwithstanding all of the above, extensive regulations are in place to control the levels of DBP's in drinking water (EPA statute: 832-F-99-062). Irrespective of the disinfectant agent used, adherence to the requirements of consumer health, producer responsibility and the regulatory authorities will remain a trade-off.

The choice of any suitable disinfectant will be dependent upon:

1. Ability to penetrate and destroy infectious agents
2. Safe and easy handling, storage and shipping
3. Absence of toxic residuals or carcinogenic compounds after disinfection
4. Affordability. (EPA: 832-F-99-062).

When the potential for DBP generation using chlorination was contrasted against that of alternative agents (Ozone, UV light, Peracetic acid and mixed oxidant generators), chlorination was found to be most holistic and cost effective strategy to safeguard the quality of drinking water (Freese and Nozaic, 2004)

It has been proposed that the most sustainable solution for controlling DBP formation remains the exclusion of inorganic and organic precursors (Trussell, 1992; White, 1992). Currently flocculation, sedimentation, coagulation and filtration or extraction with activated carbon treatments are the most effective measures that can be employed for the control of THM precursors (Krasner *et al*., 2006; Lou and Lin, 2008). However due to the incremental organic and inorganic burdens in ever diminishing raw water sources, the sustained cost effectiveness of precursor removal will remain under progressively incremental pressure.

It has been claimed that the unique attributes of the ECA technology have the capacity to generate lower levels of DBP's (Bakhir, 2003; Bakhir *et al*., 2003). This is attributed to both the lower dosages of oxidant required to achieve microbial inactivation, as well as the mixed oxidant composition of the disinfectant solution. A study was commissioned to assess DBP production when the oxidant Anolyte solution was used as a disinfectant and biofilm control intervention in dental unit water lines contaminated with mature biofilms. It was reported that the exposure of the well established biofilms to the undiluted ECA solutions resulted in THM levels well below the permissible exposure limit (PEL) of 80 µg/litre as set out by the US-EPA (Puttaiah and Siebert, 2003). However these studies are by no means exhaustive and appropriate investigations will be required to adequately detail the full implications of ECA applications and DBP production (Fenner *at al*., 2006).

As with any raw water source, the levels of Total Organic Carbon (TOC) which includes dissolved humic and fulvic acids, as well as water turbidity, will influence the outcome of any disinfection attempt. In the pursuit of a universal and cost effective biocidal potable water treatment strategy, the prospect of DBP formation will remain a constant reality and compliance with global statutes of permissible exposure limits will always be a challenge.

4.8 Corrosion

Chlorine as well as the other related oxidation products of chlorides are acknowldeged to increase the corrosion potential of susceptible metals and alloys such as stainless steel. The extensive use of the latter material in high care food and beverage processing facilities increases the risk of adverse reactions when the electrolysed oxidant solutions are advocated for cleaning and disinfection purposes. Potentiodynamic studies using the oxidant solutions of a halogenated and a non-halogenated electrolyte were assessed to establish the potential risk of pitting and crevice corrosion in AISI 304 series stainless steel. While the undiluted oxidant solution of the sodium chloride based electrolyte did reveal an increased risk of localised corrosion, no risk of equivalent corrosion was elaborated when a full-strength sodium bicarbonate derived oxidant solution was assessed (Pistorius and Biermann, 2001).

In a survey conducted on the compatibility of a diverse array of metal types exposed to electrolyzed water produced at different pH permutations, it was reported that the corrosion risk of acidic electrolysed water substantially exceeded that of electrolysed solutions produced at neutral pH (Ayebah and Hung, 2005).

It is important to discern between corrosion and rust formation, and the latter arises from the generalised conversion of ferrous to ferric ions which when reduced with oxygen results in the formation of ferric oxides with rust deposits. Localised corrosion describes the focal disruption of the passive diffusion barrier at the metal:oxide interface, the penetration of chloride ions through the impaired interface and the localised dissolution of the underlying metals through direct electron transfer reactions. At a constant chloride concentration, this process in aluminium has been shown to be largely independent of a pH effect (McCafferty, 2003).

As with other disinfectant products, it is fundamentally important to apply the compounds under the appropriate conditions with due recognition of exposure period, ambient conditions e.g. temperature, and the optimum strength of oxidant solution as would be required to achieve the required antimicrobial control. In high risk applications, it may be prudent to consider substantially diluted chloride based or non-halogenated oxidant solutions for antimicrobial and biofilm control interventions (Thantsha and Cloete, 2006).

4.9 Conclusions

The use of the ECA technology as a substitute for conventional chemical based interventions has been reported over an extensive array of applications.

However, the substantial number of different reactor types and the equally diverse array of solutions produced hinders the universal adoption of the ECA technology as being a reliable biocidal intervention with repeatable results.

Disparity of assessment technique as well as that of application protocols similarly distracts from the intrinsic value of the technology, and further assessments should be borne out of consistency of device choice, solution generation specifications as well

as application method. Similarly, laboratory technique also needs to be properly appraised as substantial differences in purported antimicrobial efficacy may be reported which may not necessarily reflect equivalent performance under field conditions.

The performance evaluations over the diverse range of applications have verified the value of the technology in most of the fields assessed. However whilst the ECA technology is described as being essentially benign in comparison to equivalent chemical analogues, the perception that the solutions can be applied without due regard for potentially adverse impacts warrants highlighting. Consideration of issues including material compatibility and effluent management must be revisited prior to the unfettered adoption of the technology.

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Chapter 5

Non-gravimetric measurement of Electro-Chemically Activated water as a biocidal assessment tool

5.1 Abstract

Current methods of evaluating the direct antimicrobial efficacy of biocidal agents have traditionally been dependent upon established bacterial culture techniques. Electro-Chemically Activated (ECA) solutions represent the aqueous products of electrolysis, wherein the gravimetric parameters of dilute salt solutions have been selectively manipulated beyond the range of theoretical physicochemical variances normally associated with conventional electrolysis. These extraordinary variances are best described by the Oxidation Reduction Potential (ORP) or REDOX potential. Serial dilutions of a selection of common gram positive and negative bacteria were exposed to a series of diluted ECA solutions. In order to distinguish between the antimicrobial effects attributed to an elevated REDOX potential and that of established chlorine based compounds, the antimicrobial efficacy of an electro-activated bicarbonate salt solution was evaluated under an equivalent dilution series with the same bacterial bioload titrations.

Both saline and bicarbonate based solutions displayed distinctive biocidal properties which were non-linear in nature. The biocidal efficacy of both highly diluted salt and bicarbonate based anolyte solutions supports the pivotal role that changes to environmental oxidation reduction potential may play in disrupting bacterial homeostasis. By applying an ECA solution of qualified ORP to the micro-environment of the bacterium and by using the measured REDOX potential as a surrogate antibacterial monitor, one is able to afford a reliable real-time measure of biocidal efficacy. It is proposed that the measurement of ORP is an effective and complementary validation tool for conventional antimicrobial assessment procedures.

5.2 Introduction

As the universal solvent, water is near ubiquitously used in the processing and production of perishable foods and beverages. Hence, few other substances have the same capacity to influence the potential risk of transmission of foodborne illnesses and premature product spoilage.

Just as accurate monitoring and recording of disinfection procedures is a critical component of any robust quality-assured safety program, so to, is the need to adequately administer and regulate the hygiene practices associated with process and/or ingredient water (Suslow, 2004).

Current water chlorination protocols are monitored by qualitative assessment of the concentrations of free and total chlorine. Standard colorimetric titration kits afford little more that an estimation of the antimicrobial ranges of active chlorine compounds that are present in the processing and production system. Notwithstanding the notorious unreliability of these qualitative assessments, the capricious antimicrobial efficacy of chlorine compounds outside of their recommended operational pH ranges further constrains the integrity of the quality assured judgement (Park *et al.*, 2004).

The universal validity for the use of free chlorine sensors as surrogate monitors of microbial inactivation has previously been disputed due to a variety of uncontrolled and directly dependent variables which influence the interpretation of the biocidal conditions (Helbling and VanBriesen, 2007).

The "Oxidation-Reduction Potential" (ORP) or REDOX as measured in millivolts, has recently been introduced as an easily standardized approach to maintaining optimal process and ingredient water quality (Suslow, 2004). ORP offers many advantages for "real time" monitoring, and the proposed correlation between REDOX potential and bactericidal effect strongly supports its use as a rapid and reliable, single value, predictive measurement parameter for the degree of microbial inactivation of the biocide treated water (Stevenson, *et al.*, 2004; Suslow, 2004; Kimbrough *et al.*, 2006). In addition, it permits the operator to assess the real-time activity of the

biocidal treatment as opposed to the standard compliance of the applied dosage of biocide (Suslow, 2004).

Historically, interventions that are designed to result in a shift in the ORP of a disinfectant solution have depended upon the calculated addition of oxidant reagents, many of which also have intrinsic biocidal capacity. These studies were thus not able to differentiate between the direct proportion of antimicrobial effect accorded to the oxidant reagent or that due specifically to the elevated ORP. The use of non-halogenated reagents to generate exaggerated shifts in the ORP of ECA oxidant solutions have confirmed the distinctive antimicrobial role that an elevated ORP level will play, independent of the presence of chemical oxidant compounds (Kimbrough *et al*., 2006).

In terms of measuring the ORP of a treated system, reliable probes, with analog and digital recording capabilities have been built into system designs, and these probes have been integrated into audible, visual and remote alarm systems in order to alert an operator of an "out-of-specification" process parameter. The primary advantage for the integration of an ORP measurement tool into the monitoring protocol for any treated water system is that it affords the operator with a rapid and single-value measure of the disinfection potential of the treated water system in any food or beverage processing facility (Suslow, 2004).

Research has shown that exposure to a solution with an ORP value of > 650mV is capable of eliminating spoilage and pathogenic bacteria such as pseudomonad's, *Salmonella spp* and *E.coli* within a few seconds. Liao *et al*. (2007) have demonstrated that progressively higher inactivation efficiencies are directly related to increasing ORP values. Available literature also confirms that progressively more advanced microbial forms such as yeasts, moulds, algae and water borne protozoa require incremental exposure periods to higher milli-volt challenges for inactivation to be complete (http://www.orpmeter.com).

A strong oxidizing agent e.g. hydrogen peroxide or ozone in contact with a viable bacterium effectively disrupts the electronic equilibrium intrinsic to the functional bacterial barrier structure thereby eventuating in a loss of vital function (Zinkevich *et*

al., 2000; Suslow, 2004). As with an oxidative stress scenario, lower ORP values i.e. < +500mV, result in bacterial inactivation or 'stasis' which may be readily reversible when more favourable conditions are restored.

It is readily acknowledged that the water treatment chemicals conventionally used in food and beverage processing systems frequently result in suboptimal disinfectant performance due to the direct shelter afforded to the microbes within the actual food or beverage products, the ubiquitous biofilm growth, as well as the design and construction inadequacies of the process infrastructure. Diligent hygiene monitoring thus remains a Critical Control Point (CCP) that will ensure the maximum disinfection potential of the treated water destined for either sanitation of in-contact process surfaces or where the same is intended for intimate product contact.

5.3 Electrochemical Activation (ECA) of water

The Electrochemical Activation (ECA) of dilute brine solutions has been reported to result in the generation of solutions with anomalous reactional abilities and behavioural characteristics (Prilutsky and Bakhir, 1997; Bakhir, 1999).

Classic electrolysis refers to the decomposition of brine to produce a variety of biocidal chemical compounds. Conversely, ECA refers to the acquisition by the water medium of uniquely distinctive properties which exceed conventional chemical transformations. In the process of the electrolytic decomposition of water, particles or compounds are formed which cannot exist outside of benign non-activated water (Bakhir, 1997; Prilutsky and Bakhir, 1997; Bakhir, 1999; Diao *et al*., 2004).

During electrochemical activation, three categories of products are proposed to be generated within the solution. These comprise:

1. Stable products - these are stable acids (in the Anolyte) and bases (in the catholyte) which influence the pH of the solution,

2. Highly active unstable products – including 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 gases, 0.2-0.5 µm in diameter and with concentrations up to 10^7 ml^{-1} which are uniformly distributed throughout the solution, and

3. Longer lasting, quasi-stable structures, which are formed at or near the electrode surface and which are comprised of free structural complexes of hydrated membranes that form around the ions, molecules, radicals and atoms. (Bakhir, 1997; Prilutsky and Bakhir, 1997).

While these categories are somewhat limited in their description, a more complete analysis of the behaviour of solute molecules at the charged electrode surface is detailed by Stumm and Morgan (1996). While the use of the oxidizing solution as a biocide has been extensively reported, the minimum effective dose of the biocide is typically represented as being a function of the concentration, exposure time and where appropriate, the disinfection hurdle, wherein a minimum point is attained where sufficient free active ingredient is available to neutralize any microbial activity to a prescribed level (Suslow, 2004).

5.4 Objective of the study

The founding hypothesis of this study was to test the contention that non-gravimetric measures of antibacterial efficacy i.e. ORP or REDOX potential may successfully be employed as a surrogate measure for quantifying the biocidal capacity of ECA solutions.

5.5 Materials and Methods

5.5.1 Generation of ECA Biocide

In contrast to the high mineralization oxidant solutions described in other reports (Shetty, *et al.*, 1999, Zinkevich, *et al.*, 2000), the halide-based oxidant biocide (S1) described in this study was generated from a 0,25% saline solution of 99.37% purity. Alternatively, the non-halide based oxidant solution (S2) was generated from a 0.3%

sodium bicarbonate feed solution of 99.7% purity. Both oxidant solution types were prepared using softened water (< 20 mg/L $CaCO_3$) derived from an anionic exchange resin. The solutions were generated by a commercial multi-reactor device installed at the Radical Waters' premises (Kyalami Business Park, South Africa).

5.5.2 Physico-Chemical titrations

After qualified dilution, the measurements of pH, ORP and Electrical conductivity (EC) were conducted for both the saline and bicarbonate derived oxidant solutions using a variety of diluent solutions of different mineralization. The ORP, EC, and pH were all measured before the solutions were used. A Waterproof double junction ORPScan of 1mV resolution was used to measure ORP of the anolyte solutions. Electrical conductivity was measured using a Waterproof ECScan of 0.01mS resolution, and the pH was measured with a waterproof pHScan 2 tester. All probes were supplied by Eutech Instruments (Singapore) and were calibrated with appropriate standards prior to solution measurement. The diluent solutions comprised municipal (tap) water, distilled water (Reitzer Pharmaceuticals, Sunninghill, SA) and de-ionised water (Food Consulting Services, Midrand, SA), and the dilution series ranged from undiluted Anolyte through dilutions of 50, 20, 10, 2 and 1%.

5.5.3 Antibacterial efficacy titration

The modified suspension test as described by Harrigan (1998) describes the addition of a known number of viable microorganisms in suspension to a solution of disinfectant (ECA oxidant) at the required concentration. The antimicrobial efficacy of the exposure is determined by whether a complete microbial inactivation was achieved and is reported as either displaying growth or no growth when plated and incubated.

5.5.4 Preparation of the cell suspension

The test organisms (*Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*) were derived from pure culture strains maintained at the

Department of Microbiology, University of Pretoria, and suspensions were prepared by growing the cells in Nutrient Broth (Biolab) for 24 h at 37°C. Two (2) ml of each cell suspension was centrifuged for 15 min at 2200 rpm. The supernatant was discarded and the pellet was re-suspended in sterile ¼ strength Ringer's solution (Merck). This cell suspension (10^9cfu/ml) was used to prepare the serial dilutions of the microbial suspensions using ¼ strength Ringer's solution (International Dairy Federation, 1962).

5.5.5 Test procedure

The experiment was designed to construct a matrix for the evaluation of the effect of a decreasing Anolyte concentration versus decreasing cell count for each bacterial species. The Anolyte at the required concentration was distributed in 9 ml quantities in sterile borosilicate test tubes. An aliquot of 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 ¼ strength Ringer's solution as a control. After an exposure period of 8 min, 0.1 ml was plated on Nutrient Agar (Biolab). The plates were incubated at 30°C, and were inspected for bacterial growth after 24-48 h. A 100% inactivation rate was recorded when no growth was visible (International Dairy Federation, 1962).

5.6 Results

5.6.1 Halide based anolyte – NaCl

5.6.1.1 Physico-Chemical titrations

Freshly generated anolyte was produced using the proprietary coaxial FEM based electrode, with an influent saline concentration of 2.5g/l, an intra-reactor flow rate of 350ml/min and a power loading of 12V and 5A. The oxidant anolyte solution was quantified in accordance with previously described post-production parameters (Table 1) (Bakhir, 1999).

Table 1. Physicochemical attributes of the undiluted saline anolyte solution.

Solution parameter	Value
Salt concentration	2,5 g/l
ORP	969mV
EC	4.15 mS/cm
pH	7.3

The saline based anolyte (S1) was diluted according to the prescribed dilution series for each different diluent type and the graphic representation of the value variations for each physicochemical parameter for each dilution are detailed in Figures 1, 2 and 3.

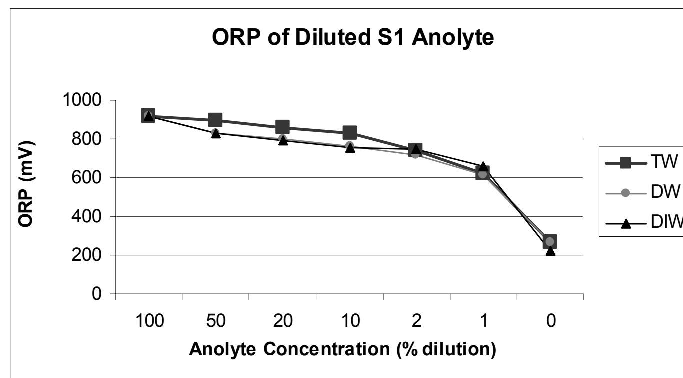

Figure 1. Changes in ORP of saline anolyte solution when diluted at different strengths and with different diluents (Legend: S1- NaCl, TW – Tap Water, DW – Distilled Water, DIW – De-Ionised Water).

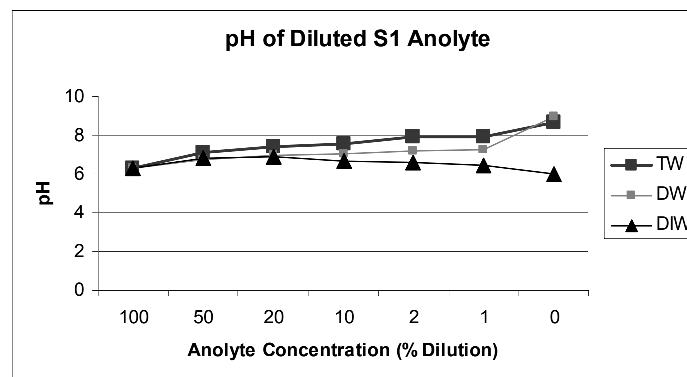

Figure 2. Changes in pH of saline anolyte solution when diluted to different strengths and with different diluents. (Legend: S1- NaCl, TW – Tap Water, DW – Distilled Water, DIW – De-Ionised Water).

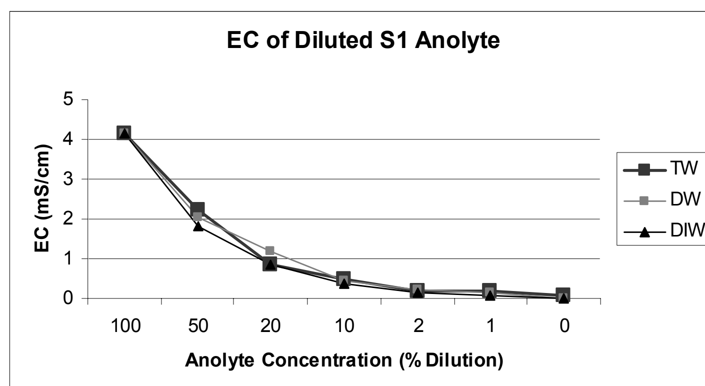

Figure 3. Changes in the Electrical Conductivity (EC) of saline anolyte solution when diluted at different strengths and with different diluents (Legend: S1 - NaCl, TW – Tap Water, DW – Distilled Water, DIW – De-Ionised Water).

As evidenced from the dilution series above, there were insignificant differences between the three diluent solutions and the physicochemical parameters of the anolyte when diluted. The progressive dilution of the saline based anolyte resulted in a non-linear reduction in the REDOX potential, while the pH of the diluted anolyte rapidly assumed the pH value of the diluent solution. The dilution of the anolyte resulted in a near linear reduction trend of the electrical conductivity of the diluted solutions.

5.6.1.2 Antibacterial efficacy titration

A series of logarithmic dilutions of NaCl based anolyte was prepared and the variations in physicochemical parameters for each dilution were recorded (Table 2).

Table 2. Physicochemical parameters of the serial dilution of the NaCl anolyte solutions.

Dilution	pH	EC	ORP
Neat	6.60	5.54	972
1:10	6.13	6.76	836
1:50	6.64	6.81	742
1:100	6.87	6.86	587
1:1000	6.92	6.84	461
1:10 000	7.13	6.92	442
Diluent*	7.08	6.89	451

* ¼ strength Ringer's solution was used as the diluent.

5.6.1.3 Antibacterial efficacy

Suspensions of the different bacterial strains were serially diluted to predetermined cellular concentrations and were exposed to the range of anolyte dilutions as described above. After plating and incubation, the presence of bacterial growth was recorded and the results are tabulated per bacterial species and were detailed as either growth or no growth (100% inactivation).

Table 3. Inactivation of *B. subtilis* over a range of saline anolyte dilutions.

Analyte concentration	Cell concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	Growth	Growth	No growth	No growth	No growth
1:50	Growth	Growth	Growth	No growth	No growth
1:100	Growth	Growth	Growth	No growth	No growth
1:1000	Growth	Growth	Growth	No growth	No growth
1:10 000	Growth	Growth	Growth	Growth	No growth

While the undiluted anolyte achieved inactivation of all bacterial challenges, it was only when the challenge dose was reduced to a $\log_{10} 4$ count that the 1:10 dilution of saline based anolyte resulted in complete inactivation. Further reductions in the challenge dose resulted in total bacterial inactivation even at higher anolyte dilutions.

Table 4. Inactivation of *S. aureus* over a range of saline anolyte dilutions.

Analyte concentration	Cell concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	No growth	No growth	No growth	No growth	No growth
1:50	No growth	No growth	No growth	No growth	No growth
1:100	Growth	No growth	No growth	No growth	No growth
1:1000	Growth	Growth	Growth	No growth	No growth
1:10 000	Growth	Growth	Growth	No growth	No growth

Complete inactivation of high dose *S. aureus* challenge was achieved up to a 1:50 anolyte dilution and the microbial inactivation rate described an inverse linear trend with increasing anolyte dilution thereafter. Complete inactivation of all cell suspensions with counts of 3 log_{10} and below was achieved with anolyte dilutions of up to 1:10 000.

Table 5. Inactivation of *E. coli* over a range of saline anolyte dilutions.

Anolyte concentration	Cell concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	No growth	No growth	No growth	No growth	No growth
1:50	Growth	No growth	No growth	No growth	No growth
1:100	Growth	Growth	Growth	No growth	No growth
1:1000	Growth	Growth	Growth	Growth	Growth
1:10 000	Growth	Growth	Growth	Growth	Growth

Aside from the undiluted anolyte solution, inactivation of the highest concentration of *E.coli* organisms was only achieved up to a 1:10 strength anolyte solution. No inactivation of the bacterial cells in suspension was achieved with anolyte dilutions of 1:1000 or higher.

Table 6. Inactivation of *Ps. aeruginosa* over a range of saline anolyte dilutions.

Anolyte concentration	Cell concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	Growth	No growth	No growth	No growth	No growth
1:50	Growth	No growth	No growth	No growth	No growth
1:100	Growth	No growth	No growth	No growth	No growth
1:1000	Growth	Growth	Growth	No growth	No growth
1:10 000	Growth	Growth	Growth	Growth	Growth

While the undiluted anolyte displayed total inactivation of all *Ps. aeruginosa* organisms, no further inactivation of the high bacterial cell count preparations was

achieved with the diluted anolyte solutions until the suspension had been diluted to a 5 $\log_{10}$ cell count. At this cell concentration, the 1:100 anolyte dilution was still able to achieve a complete inactivation of all bacteria. No inactivation was obtained even at low cell suspension counts when the anolyte solution was diluted beyond 1:1000.

5.6.2 Non-halide based anolyte – $NaHCO_3$

5.6.2.1 Physico-Chemical titrations

A 3g/l $NaHCO_3$ solution of 99.7% m/m purity grade was used in the preparation of the non-halide based anolyte. This solution was activated using the customised sodium bicarbonate (S2) ECA device. The intra-reactor flow rate was preset to 350ml/min and the power supply to the reactor was increased to 24V and 3A relative to that of the saline based generation. The characteristics of the solution were measured in accordance with previously described conventions (Table 7).

Table 7. Physicochemical attributes of the undiluted sodium bicarbonate anolyte solution.

Solution parameter	Value
$NaHCO_3$ concentration	3 g/l
ORP	958mV
EC	1.36 mS/cm
pH	6.88

Relative to the saline based anolyte of reduced mineralization i.e. 2.5g/l, there was a slight reduction in REDOX potential and a substantially reduced electrical conductivity of the bicarbonate derived anolyte solution. While the ionic dissolution of the bicarbonate salt has been shown to be substantially more temperature dependent than that of NaCl, both feed salt solutions were prepared with water at ambient temperature i.e ± 20°C, and the reduced EC is a direct reflection of the diminished electrical reactivity of the dissolved bicarbonate salt in solution.The bicarbonate derived anolyte was diluted according to the prescribed dilution series and the graphic representation of the variations in physicochemical parameters for each dilution are presented in figures 4, 5 and 6.

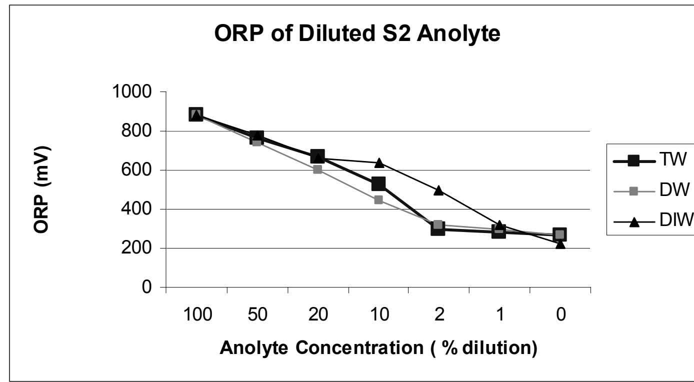

Figure 4. Changes in anolyte ORP when diluted at different strengths and with different diluents (Legend: S2 - $NaHCO_3$, TW – Tap Water, DW – Distilled Water, DIW – De-Ionised Water).

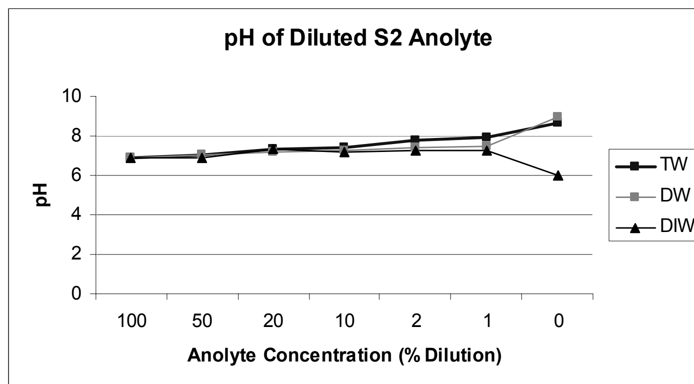

Figure 5. Changes in anolyte pH when diluted to different strengths and with different diluents. (Legend: S2 -$NaHCO_3$, TW – Tap Water, DW – Distilled Water, DIW – De-Ionised Water).

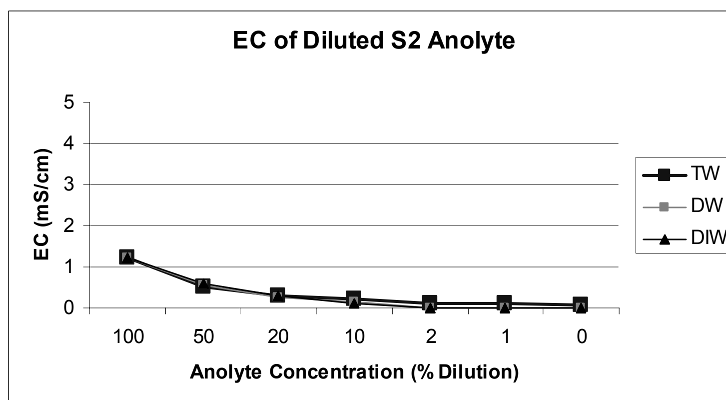

Figure 6. Changes in the Electrical Conductivity (EC) of anolyte solutions when diluted at different strengths and with different diluents (Legend: S2-$NaHCO_3$, TW – Tap Water, DW – Distilled Water, DIW – De-Ionised Water).

The changes in the physicochemical parameters of the sodium bicarbonate anolyte displayed substantial deviations over the dilution series relative to that of the saline based anolyte. The reduction in REDOX potential of the $NaHCO_3$ based anolyte occurred more rapidly and the ORP fell below the proposed antibacterial hurdle value of 600mV at approximately 15% strength versus that of a 1% strength detailed with the NaCl based anolyte. The pH of the diluted anolyte rapidly equilibrated with the pH of the diluent solution, while the EC described a linear reduction albeit from a significantly lower initial conductivity value.

5.6.2.2 Antibacterial efficacy titration

As with the NaCl derived anolyte, an equivalent series of logarithmic dilutions of the $NaHCO_3$ based anolyte were prepared and the variations in physicochemical parameters for each dilution were recorded (Table 8).

Table 8. Changes in physicochemical parameters of the diluted Sodium Bicarbonate anolyte solutions.

Dilution	pH	EC	ORP
Neat	7.63	1.36	958
1:10	8.17	5.45	842
1:50	7.99	5.51	784
1:100	6.73	5.73	468
1:1000	7.23	5.01	386
1:10 000	7.18	5.69	377
Diluent*	7.75	5.77	444

* ¼ strength Ringer's solution was used as the diluent throughout this study.

4.6.2.3 Antibacterial efficacy

The serial dilutions of the bacterial strains were prepared and exposed to the $NaHCO_3$ anolyte in accordance with the previously described protocol, and the same evaluation and recording procedure was followed.

Table 9. Inactivation of *B. subtilis* over a range of sodium bicarbonate anolyte dilutions.

Anolyte concentration	Cell concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	No growth	No growth	No growth	No growth	No growth
1:50	Growth	Growth	No growth	No growth	No growth
1:100	Growth	Growth	No growth	No growth	No growth
1:1000	Growth	Growth	Growth	Growth	Growth
1:10 000	Growth	Growth	Growth	Growth	Growth

Bicarbonate based anolyte was effective in achieving complete inactivation of all *B. subtilis* cell suspensions up to a 1:10 dilution. Solutions diluted below 1:10 strength were only effective in inactivating cell suspensions of $4\log_{10}$ and below and there was incomplete inactivation of all suspensions when the anolyte was diluted to 1:1000 and more.

Table 10. Inactivation of *S. aureus* over a range of sodium bicarbonate anolyte dilutions.

Anolyte concentration	Cell Concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	No growth	No growth	No growth	No growth	No growth
1:50	No growth	No growth	No growth	No growth	No growth
1:100	No growth	No growth	No growth	No growth	No growth
1:1000	Growth	Growth	Growth	No growth	No growth
1:10 000	Growth	Growth	Growth	Growth	Growth

As with the exposure tabulation of the saline based anolyte dilutions, the bicarbonate based anolyte was effective in inactivating all cell suspensions beyond a 1:50 dilution. It would thus appear that *S. aureus* is equivalently sensitive to exposure to both saline and bicarbonate based anolytes even at substantial dilutions. This matched antibacterial efficacy is achieved without the addition of recognized halogen derived

biocidal compounds in the diluted bicarbonate based anolyte solutions. However, given that ¼ strength Ringers was used as the diluent solution, it is feasible that some of the constituent chloride ions (1.358gm/l or 1358ppm) may have undergone transformation into reactive chlorine species following admixture with the bicarbonate based anolyte.

Table 11. Inactivation of *E. coli* over a range of sodium bicarbonate anolyte dilutions.

Anolyte concentration	Cell Concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	No growth	No growth	No growth	No growth	No growth
1:50	No growth	No growth	No growth	No growth	No growth
1:100	No growth	No growth	No growth	No growth	No growth
1:1000	Growth	Growth	No growth	No growth	No growth
1:10 000	Growth	Growth	Growth	Growth	Growth

Aside from the 1:10 000 dilution, the bicarbonate based anolyte was effective in achieving inactivation of all *E.coli* suspensions with bacterial counts of up to 4 $\log_{10}$. The diluted anolyte solutions were effective in the inactivation of bacterial counts of 6 $\log_{10}$ and below using a 1% anolyte concentration, and this corresponded to an ORP of the diluted anolyte of 468mV (Table 8).

Table 12. Inactivation of *P. aeruginosa* over a range of sodium bicarbonate anolyte dilutions.

Anolyte concentration	Cell concentration (cfu/ml)				
	10^6	10^5	10^4	10^3	10^2
Control	Growth	Growth	Growth	Growth	Growth
Undiluted	No growth	No growth	No growth	No growth	No growth
1:10	No growth	No growth	No growth	No growth	No growth
1:50	No growth	No growth	No growth	No growth	No growth
1:100	No growth	No growth	No growth	No growth	No growth
1:1000	Growth	Growth	Growth	Growth	Growth
1:10 000	Growth	Growth	Growth	Growth	Growth

As with table 11 above, the bicarbonate based anolyte achieved complete inactivation of all cells in suspension up to a 1% concentration of bicarbonate derived anolyte. No inactivation was achieved when the suspensions were exposed to anolyte dilutions of 1:1000 and above.

5.7 Discussion

From the coordinate plots detailed in Figures 1 and 4, it is evident that the changes in the ORP of the diluted solutions do not describe a linear trend when contrasted against the incremental dilution percentage. While there was a direct correlation between the Anolyte dilution series and the proportionate reductions in Electrical Conductivity (EC) (Figures 3 and 6) arising from an anticipated linear dilution effect, the same correlation could not be ascribed to the non-linear trend detailed when the REDOX potential was measured over the equivalent dilution series. There appears to be a mismatched, yet directly causal relationship between the dilution effect on the linear parameters (concentration of chemical constituents) and the non-linear effects on the REDOX potential when contrasted against the capacity to predict the irreversible damage to the different bacterial strains.

The data from the two antibacterial titration series confirms that the non-halide based Anolyte was effective as a broad spectrum antibacterial agent against most of the test bacteria, with the qualified exception of *B. subtilis*, which at the highest bacterial cell concentration was not inhibited at an Anolyte dilution which exceeded 1:10. In contrast, all other test microorganisms with titres of up to 6 $\log_{10}$ were inhibited with Sodium bicarbonate based Anolyte when diluted up to a final exposure concentration of 1%.

Contrary to the conventional dogma which draws a correlation between specific bacterial susceptibility and a specific concentration of biocidal compound (i.e. MIC), the antibacterial efficacy of the Anolyte as an biocidal agent appears not to be predicatated upon by the type of cell barrier structure of the test microorganisms (Gram negative vs. Gram positive), but rather the data suggests that the antibacterial response tends to be strain specific. While by no means exhaustive, it is feasible to speculate that bacteria with an intrinsic capacity for endospore formation (*B.subtilis*)

as well as EPS formation (*Ps.aeruginosa*), may well display a non-adaptive and adjunct capacity to withstand the adverse stressors that the exposure to an overtly oxidative milieu may elicit.

It is conventionally recognised that the classical chemical biocide model and its associated gravimetric antibacterial efficacy titrations will elaborate a repeatable 'Minimum-Inhibitory Concentration' (MIC). This MIC measure subscribes to a conventional linear dilution model which predicts the minimum concentration of an active biocidal ingredient that would be required in order to eliminate a prescribed bacterial bioload within a predictable time frame. However, when the Anolyte solutions are subjected to an equivalent dilution series, the progressive dilution of the biocidally active chemical constituents as reflected in the EC dilution series (Figures 3 and 6), do not correspond with the hithertofore acknowledged levels of active chemical ingredient i.e. MIC which are conventionally reported as being a prerequisite for antibacterial compliance.

The distinctly elevated levels of REDOX potential suggests a causal relationship with the degree of bacterial inactivation, and the resultant biocidal effect does not appear to be dependent upon the proportionate concentration of conventionally recognised biocidal agents i.e. halide based reagents. This tentative causal relationship has also been proposed by Kim *et al*., (2000), Park *et al*., (2004) and Kimbrough *et al*., (2006). The measurement of the REDOX potential of the progressively diluted anolyte solutions thus appears to be a reliable non-gravimetric measure for the prediction of its antibacterial efficacy, and it is proposed that the predictability of its composite antibacterial attributes may be substantially augmented were it to be integrated with the roles that pH and electrical conductivity may play in overall biocidal effect.

Traditional antibacterial efficacy assessment protocols call for a procedurally qualified and quantified sampling of biocide treated environments in order to establish the success of the bacterial control strategy. Such prescriptive procedures and their allied protocols are seldom designed to afford blanket representation of highly variable environments which are likely to be contaminated with different bacteria. Similarly, and notwithstanding the vagaries of individual sampling techniques and the spectrum of other environmental features i.e. temperature, time, sampling asepsis etc.,

that may impact upon the relevance of a sampling effort, bacterial cultures also require extended periods of incubation under highly specific culture conditions prior to any definitive indication being afforded as to the levels of residual bacterial contamination that may follow an antibacterial intervention.

While this study readily acknowledges the indispensability of conventional sampling and culture procedures, the time constraints allied to the delays in the availability of results especially with highly specific culture conditions, further reaffirms the limits that conventional sampling and culture protocols impose on optimum productivity goals. In light of the relative novelty of this approach for assessing antibacterial potential, it would be reckless to promote the exclusive adoption of the REDOX monitoring technology without the retention of established and complementary bacterial control and assessment measures as are currently accepted and practiced globally. However, despite this novelty, quality assurance managers now have access to a tool whereby it is possible to control water and product disinfection on a real-time and in-process basis without having to rely exclusively upon a deferred laboratory based result which is constrained by the limitations of conventional microbial sampling and culture protocols.

5.8 Conclusion

Traditional conventions for bacterial control of process and potable water in food or beverage production are dependent upon a basket of features, wherein the goal of minimizing the effective dose of the sanitizer used for bacterial disinfection is balanced against the adverse dose dependent implications on product sensory quality, the environment and crucially, human health.

Aside from confirming the safe and novel biocidal capabilities of the ECA solutions, of greater significance was the quantification of the intrinsic measurable capacity of the ORP of diluted ECA solutions as being a reliable tool to predict the biocidal effect of a given Anolyte dilution when dosed into a system with a projected bacterial bioload. The study provides evidence to support the contention that Electro-Activated water solutions may exert their antibacterial effect in a manner which is distinctive from that reported for conventional chemical based remedies. That bacterial

inactivation could be achieved at significant dilutions of both of the two types of anolyte solutions reaffirms the hypothesis that the antibacterial effect is unlikely to be associated with a purely gravimetric measure of biocidal reagent. Additionally, the exclusion of reactive halogen based species from the antibacterial efficacy appraisal through the use of a Sodium Bicarbonate derived anolyte, substantially confirmed the antibacterial effect of the non-halogen reagent.

In conclusion, the measurement of an array of anomalous physicochemical attributes of the ECA solutions on a real-time and in-process basis may provide a reliable tool to describe optimal antibacterial concentrations in different process water systems, and consequently may permit its users to enhance quality assurance measures and decisions without compromise to product quality or consumer satisfaction.

5.9 Acknowledgements

S. Malherbe for the details of the anolyte efficacy titration assays, and R. Bagnall for assistance with the use-dilution graphics.

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Chapter 6

Evaluation of the biocidal effects of ECA solutions using Atomic Force Microscopy (AFM)

6.1 Abstract

Reports in the literature attest to the ultra structural damage to bacterial cell wall structures that follow exposure to Electro-Chemically Activated (ECA). Atomic Force Microscopy (AFM) is a high-resolution, three dimensional imaging technique, which facilitates both the qualitative and quantitative assessment of the effects of biocidal interventions at the ultra-structural level.

The use of the electrolyzed oxidizing Anolyte (Actsol®[1]) solution as a biocide is well established, and this study was designed to assess the morphological alterations that exposure to Actsol® solutions would cause in a variety of bacterial strains under controlled conditions. Four mixed bacterial strains were exposed to the Actsol® solution produced from a saline solution (0,25%) for 5 minutes and contrasted against the AFM images from equivalent, non-Actsol® treated preparations. All Actsol® treated bacterial samples revealed significant morphometric deviations relative to the untreated controls, and these changes comprised altered geometric contour, swelling, and lysis with leakage of intracellular contents. Additionally, the AFM technique afforded the further capacity to describe distinctive ultra-structural changes to cell wall morphology following exposure to the aqueous oxidizing solutions.

This study confirms the cellulolytic properties of the Electro-Chemically activated Anolyte solutions as well as the merits of employing the AFM high-resolution technology to describe bacterial morphology and the capacity to assess the ultra-structural changes that follow exposure to surface active biocides.

1- Actsol® is the registered trademark of Radical Waters (Pty) Ltd.

6.2 Introduction

The biocidal capacity of the Electrolyzed oxidizing saline solutions with anomalous physicochemical attributes generated during the Electro-Chemical Activation of dilute brine solutions has been reported extensively (Prilutsky and Bakhir, 1997; Bakhir, 1999; Marais and Brözel, 1999; Selkon *et al*., 1999; Shetty *et al*., 1999; Tapper *et al*., 1998; Marais, 2000[a,b]; Zinkevich *et al*., 2000; Diao *et al*., 2004 Nakajima *et al*., 2004 and Liao *et al*., 2007). These reports of biocidal efficacy are substantiated by a recent study wherein a non-saline electrolytic oxidant solution was also reported also reported to display substantial biocidal capacity (Jeong *et al*., 2006).

The large diversity of cell barrier architecture reflects a variety of specific adaptations to environmental and ecological pressures. Microbial surfaces are the critical interface with the external environment, and as such they play a vital role in determining cellular shape and the maintenance of turgor pressure (Dufrene and Müller, 2005). To date, most evaluations of bacterial surface morphology have relied upon 2-Dimensional images generated from optical and electron microscopy. Refinements to the preparation technique have yielded additional information on topographical features, and cryo-methods tied to electron microscopy have been able to offer the opportunity to view surface images under conditions more representative of their native state. However, these methods are substantially onerous in terms of sample preparation, analysis and are prone to artefactual change.

Aside from the limitations of specific sample preparation, an understanding of the structure, properties and functions of microbial surfaces is limited by the availability of sensitive, high resolution surface analysis techniques (Dufrene and Müller, 2005). Historically, magnification of microbial surface features with optical instruments has been limited by the wavelength of visible light and generally only offers a resolution down to 0.5 microns.

The use of electron and ion beams in a Scanning Electron Microscope (SEM) permits the resolution of images to approximately 30 angstroms. However the SEM is often destructive and does not readily yield quantifiable information on the surface topography of the sample. The use of Transmission Electron Microscopy (TEM) does

permit the imaging of intracellular structures and in particular the cytoplasmic membrane and is particularly useful for comparative microscopic studies (Joeng *et al.*, 2006).

More recently, Scanning Probe Microscopy (SPM) has been used for imaging and metrology applications to provide quantitative, 3-Dimensional topographic information of sample surfaces. As a specific refinement of the SPM technology, Atomic Force Microscopy (AFM) can provide a 3-Dimensional image of biological cells under physiological conditions with an unprecedented resolution. The unique capability of AFM to characterise the height dimension permits direct visualisation of 3-D images of structures with resolutions ranging from subangstroms to microns (Wickramasinghe, 1989; Zhou and Christie, 1995). AFM affords the potential to describe surface morphology without the constraints of vacuums and destructive staining procedures which are known to adversely impact upon the generation of artefact free images (Bolshakova *et al.*, 2001). In addition, the technology also permits the determination of the physical properties and molecular interactions at the microbial barrier interface, and thus provides fundamental insights into the structure and functional relationships of the components of cell surfaces (Dufrene and Müller, 2005).

Chemically derived characterisations of cell wall attributes have been complemented with AFM images wherein the manipulation of the charge of the cell surface using variations in concentration of monovalent ions, have confirmed that the OmpF porin in the cell wall has a nett negative charge (Dufrene and Müller, 2005).

In terms of describing some of the distinguishing structural features that are specific to different physiological states, it is recognised that adhesion is substantially dependent upon the structure and physicochemical properties of the bacterial surface. It is through the AFM imaging of bacteria under physiologically optimum conditions, that the differentiated structure, chemical composition and physicochemical properties of the surfaces of microbes can be aligned to the mechanisms that facilitate microbial adhesion and aggregation (Schaer-Zammaretti and Ubbink, 2003).

In the quest for data and images of microbes under natural artefact-free conditions, the scanning probe technology represents a significant advantage over conventional structural research techniques. In addition, the AFM technology permits sample imaging in liquids without the impediments normally associated with sample drying and the in-situ imaging procedures readily permit the direct observation of the effects of drug interventions (Bolshakova *et al*., 2001).

In Contact Mode AFM, the samples are mounted on a piezoelectric scanner which permits the 3-D manipulation of the image under high resolution. The topographical detail of the sample is translated from the electronic response of the van der Waal's forces of attraction between the tip of the cantilever and the sample which is monitored and recorded (Dufrene and Müller, 2005). As the probe tip scans, the varying topographic features result in deflections between the cantilever tip and the sample. A light beam from the laser is directed at the cantilever and reflected onto a four-sectioned photodetector. The amount of deflection of the cantilever, or the force it applied to the sample, is then calculated from the difference in light intensity on the four different photodetector sectors (Zhou and Christie, 1995).

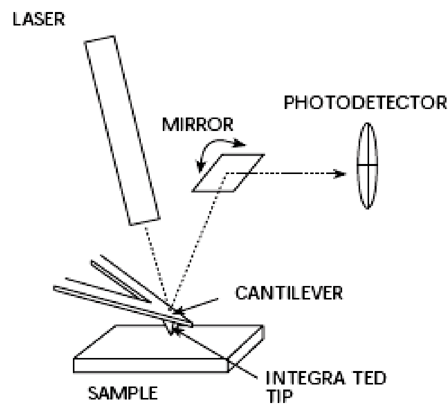


Figure 1 Graphic representation of the image generation pathway with AFM (Zhou and Christie, 1995).

Sample preparation and immobilisation of cells onto surfaces is critical for optimal imaging with the AFM technology (Doktycz *et al*., 2003). Samples must be securely attached to a solid support in order to resist the lateral forces that may be exerted by the scanning tip. More importantly, the biological object should not change their position while being scanned. While microbial cells are more suited to AFM studies

than animal tissues, the rigid spherical or rod-like structures of bacteria translate into a relatively small cell to substrate contact area (Dufrene and Müller, 2005).

Despite the relative user-friendliness of the technology, basic preparation of the sample is fundamental to the success of the imaging procedure. A crucial prerequisite for the successful and reliable biological application of AFM is that the samples have to be securely attached to the solid substrate using appropriate, non-destructive methods (Doktycz *et al.*, 2003; El Kirat, *et al.*, 2005). While there are a number of substrates available for mounting biological samples, they must all be atomically flat so that the surface of the substrate will not impact upon the natural topography of the adherent sample (Zhou and Christie, 1995).

Biological samples can be imaged in either wet or dry environments. Despite the problems associated with wet imaging, it affords the opportunity to examine the sample under natural conditions. In addition, it excludes the likelihood of artefact development due to preparation technique and it affords greater microscopic resolution (Zhou and Christie, 1995; Bolshakova *et al.*, 2001). As a dynamic system, wet imaging has been also been used to describe the real-time responses of bacterial cells by way of time-lapse photography following exposure to ECA solutions (Tapper *et al.*, 1998).

In contrast to optical or electron microscopy which are subject to complex diffraction or electromagnetic interference, SPM images are largely indifferent to variations in the optical or electronic properties of samples and will readily afford a true reflection of surface topography. However, despite the relative ease and simplicity with which AFM images can be interpreted, they are still subject to artefacts which are intrinsic to the technology. These include tip convolution where the shape and sharpness of the probe tip is mismatched to the topographical features of the sample, and feedback artefacts where background noise generated from extreme topographic features result in ghosting and exaggerated shadow effects (Zhou and Christie, 1995). These artefacts are easily recognised and can be readily remedied by repeating the scan and making changes to the settings of the area of the scan, its speed and direction.

In order to tether biological molecules to supporting surfaces, several procedures have been introduced to promote the formation of covalent bonds between the apposing surfaces. While this methodology may offer a more secure fixture of the sample, its extensive preparation increases the likelihood for the development of aberrations. Air drying and chemical fixation are used to promote cell attachment but are recognised to result in significant denaturation of the specimen (Dufrene and Müller, 2005). Bacterial images of samples derived from culture plates and imaged in minimal media were shown to be comparable to images derived from liquid media under AFM. In all cases the bacteria maintain a hydrated appearance with no evidence of collapse of the cell wall (Doktycz *et al.*, 2003).

Significant differences in vertical dimensions arise from the different preparation procedures, and the height of bacteria imaged in water is substantially greater than that of images secured under air. Additionally flagella structures are more readily imaged in air dried samples versus liquid images. Imaging in water as opposed to air reveals many different topographic features and it is proposed that the loss of resolution of the superficial topographical features may be due to hydration of the carbohydrate chains of the lipopolysaccharide moiety of the cell membrane (Doktycz *et al.*, 2003). With air imaging, the inherent features of bacterial cell walls e.g. filaments etc, become collapsed on the cellular surface and thus create a strain specific topography (Bolshakova *et al.*, 2001).

6.3 Objectives

The objectives of this study were to describe the degree of difference in microscopic change that may arise between different microbial strains following exposure to the oxidant ECA solutions, and to contrast any changes in outer barrier structure against untreated controls. Additionally, the changes in cell structure following exposure to different dilutions of the oxidant ECA solutions were compared in order to evaluate a potentially dose dependent effect.

6.4 Material and Methods

6.4.1 AFM imaging

The Atomic Force Microscopy (AFM) imaging studies were carried out with a Discoverer TopoMetrix TMX2000 Scanning Probe Microscope (Topometrix Corporation, Essex, UK).
Sample preparations were dehydrated and imaged under air. The scanner had a maximum x,y translation capability of 7 x 7 µm. In accordance with previously reported studies (Tapper *et al.*, 1998), the microscope was operated in the contact mode and graphical output was displayed on a monitor with a resolution of 200 lines x 200 pixels. Where necessary, images were enhanced by means of the plane-fitting mode, and shading was used to enhance topographic features where appropriate. The image capture, manipulation and enhancement was facilitated with the complementary software supplied by the Topometrix Corporation and Veeco, Thermo-microscopes (California) (Zhou and Christie, 1995; Symanski, 2004).

6.4.2 Preparation of microbial samples

The bacterial strains comprised type cultures that were maintained on semi-solid agar. These strains were comprised of *Staphylococcus aureus*, *Bacillus subtilis var. niger*, *Escherichia coli*, and *Pseudomonas aeruginosa*, and were plated out for both purity and viability checks. Prior to harvesting, all strains were plated out on Mueller-Hinton 1 Agar Plates (Selecta media) and were incubated at 37°C for 24 hours.

Samples of the bacterial cultures were taken with a flamed metal loop from established plaques on the agar plates. These samples were suspended in either 10ml of sterile hypotonic (2.5g/l) saline solution or an equivalent volume of either undiluted or 1 in 10 strength saline based Actsol®, the latter having been diluted with the same hypotonic saline as used to suspend the untreated control samples. These cultures were homogenized in the hypotonic saline using a mechanical agitator (Vortex V-1, Boeco, Germany) for 30 seconds. A 1 µl drop of the untreated and Actsol® exposed bacterial suspensions was then pipetted onto a silicone disc (25mm^2) which had been pre-cleaned with 70% m/v alcohol. The droplet was then spread with a flamed metal loop to a diameter of approximately 2-3mm.

Initial attempts to image the bacterial preparations on glass slides proved problematic, and silicon wafers were adopted as the standard substrate for the duration of the study. In contrast to glass slides, the use of silicon also enhanced the location of the samples through the heightened optical contrast between the polished surface and the bacterial preparations. This approach concurs with previous reports that indicate that silicon oxide wafers are effectively comparable to glass, and aside from price, are the preferred substrate (El Kirat *et al*., 2005).

While the adherence of biological materials to a substrate may be facilitated by covalent bonding to an active amine coating of the substrate, this procedure proved unnecessary as the samples readily adhered to the surface of the silicone wafers with passive dehydration. The silicon mounted preparations were air-dried for up to 10 minutes in a commercially available incubator (Labcon, S.A.) preset to 25°C.

6.4.3 Generation of ECA solutions

While it is now widely accepted that oxidant solutions produced by different ECA generation systems differ substantially relative to their final composition and activation status (Sampson and Muir, 2002), this study made use of the ECA solutions derived from the FEM-3 based technology as previously described (Bakhir, 1997, Prilutsky and Bakhir, 1997, Zinkevich *et al*., 2000).

Depending on the specific ECA generation parameters employed, a diverse array of chemical compounds can be produced. Predominantly due to the design limitations of the first and second generation reactors as well as the relative novelty of the technology being evaluated, the earlier reports detail assessments which were conducted with largely uncontrolled solutions whose repeatability of quality and hence biocidal capacity, remain questionable.

In contrast to a previous AFM study which used an earlier generation of similar reactor design i.e. FEM 2, and a saline feed solution of 5N NaCl activated at a flow rate of 800ml/minute (Tapper *et al*., 1998), the oxidant ECA solution used in this study i.e. Actsol®, was generated from a 0,25% saline solution, and the effluent solution was pH adjusted to yield a REDOX potential of +850 ± 15mV using a

standard silver-chloride electrode, an Electrical Conductivity (EC) of 5.0 ± 0.5mS/cm and a pH of 7.0 ± 0.2. The solution was generated with a prototypal device comprising two parallel FEM 3 electrolytic reactors developed by Radical Waters (Kyalami, South Africa), and the final solution quality was preset against a saline flow rate of 350 ml/minute per reactor unit.

6.5 Results and Discussion

Previous studies (Tapper *et al*., 1998; Zinkevich *et al*., 2000) have reported that the exposure of *E.coli* bacteria to the Electro-Chemically Activated oxidant solution resulted in an initial distortion of relative cellular proportions, a progressive extracellular accumulation of cytosolic content, and terminally, a universal cell lysis wherein no intact cells were evident after 5 minutes of exposure to the oxidant solution. The substantial enlargement in most cell sizes and the incremental accumulation of extracellular cytoplasmic debris as reported was substantially confirmed with the findings of this study.

While the gram positive microbial strains i.e. *S. aureus* and to a lesser extent, *B. subtilis* appeared to retain their pre-treatment morphological dimensions following Actsol® exposure, the relative dimensions of the gram negative *E.coli* and *Ps. aeruginosa* cells both displayed substantial dimensional deviations relative to the morphology of their untreated controls. In addition, the changes in cell morphology of the gram negative bacterial strains was paralleled by a substantial accumulation of extracellular debris. Of all the bacterial strains evaluated, the *E. coli* cells displayed the greatest change in cellular dimension and thus volume, and this was reflected by the substantive distortion to the relative linear proportions obtained after exposure to the Actsol® challenge.

The bacterial samples prepared directly from agar plates and imaged in air, displayed an increase in ultrastructural detail and a distinctly different overall morphology. It is proposed that these structural differences may be due to changes in the cell rigidity arising from the reduced hydration status of the cell wall. In addition, certain microbial strains imaged under air after sample dehydration were reported to develop

a scalloped shape with raised edges and these structural changes are attributed to the direct effects of dehydration (Doktycz *et al.*, 2003).

This finding is supported by Bolshakova *et al.* (2001) who report that drying of samples led to the formation of two distinctive types of artefacts – a decrease in cellular height as well as the appearance of a distinctive surface patterning.

Of all the strains included in the study, consistent evidence of these distinctly anomalous features were only observed in the images of the untreated *P. aeruginosa* preparations when viewed with both the contrast enhanced 2-D and 3-D rotational image manipulations (Fig 2). Most cells displayed a flattening along the longitudinal contour as well as the presence of punctuate depressions on the surface (Fig 2). Similar evidence of probable preparation artefacts are evidenced in SEM images as reported by Marais (2000) (Fig 4).

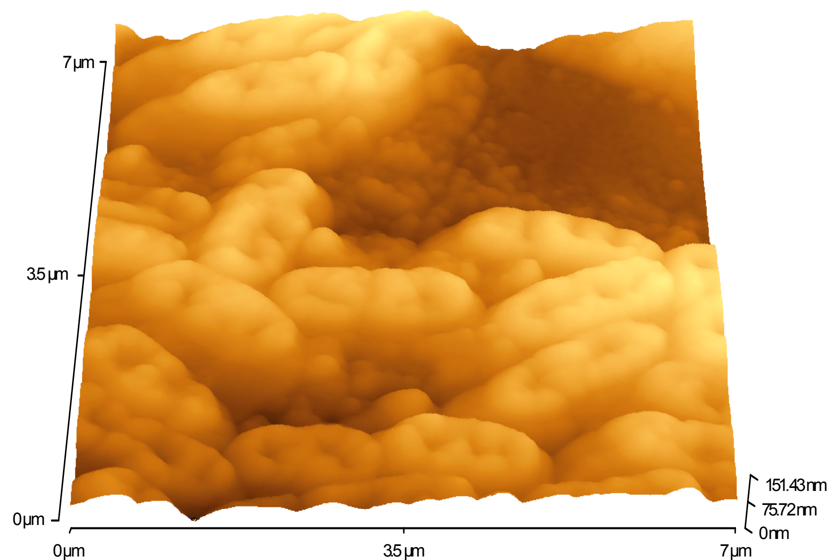


Figure 2. Distinctive punctate topography of untreated *Pseudomonas aeruginosa* cells imaged after fixation with passive dehydration.

6.5.1 Image processing – 2D colour mapping.

By editing the image with the 2D colour mapping adjustment tool, it was possible to enhance the sharpness of the image and afford a qualitative augmentation of the image. By varying the distribution of the colour intensity between the height and

shading data, it was possible to adjust the definition of the superficial image albeit with a sacrifice to the clarity of the depth perspective. Additionally, by increasing the surface reflectivity it was possible to enhance the sharpness of the 2-D image (Symanski, 2004). It has been reported that bacterial suspensions applied to the surface of mica or polished silicon oxide substrates will form flat patchwork monolayers whereby individual cells can be readily visualised (Bolshakova *et al.*, 2001).

A further benefit to this passive adhesion technique is that the preparation results in a clean AFM image which did not require any further rinsing of the samples to remove potential contamination. While this first order monolayer alignment was consistently evident in the images of the untreated *P. aeruginosa* preparations (Fig 3), the spherical dimensions of *S. aureus* resulted in a default quasi-monolayer configuration which also permitted the visualisation of separate cell topography (Fig 5). Similarly while all the AFM samples of the different bacterial strains were prepared identically, none of the untreated *E.coli* or *B subtilis* preparations displayed any predictable first order cellular alignment.

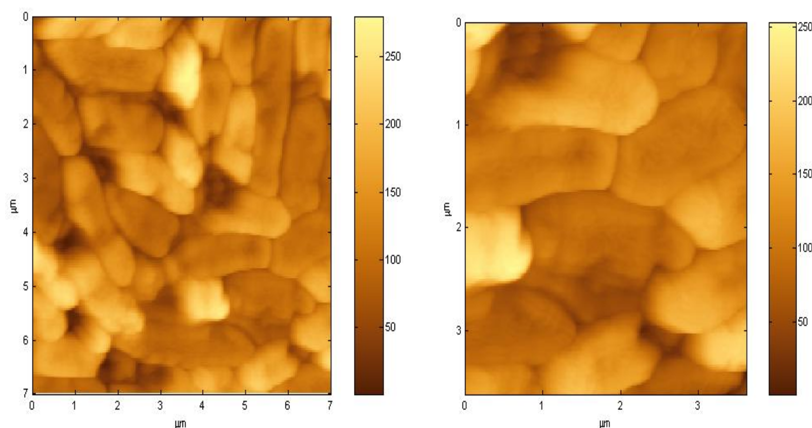


Figure 3. Two magnifications of a monolayer of untreated *P. aeruginosa* cells displaying a 'cobblestone paving' pattern (colour contrast scale displayed at the right).

Aside from the enhancement to the clarity and sharpness of the image, this manipulation also permitted the direct comparison with images generated with other ultrastructural 2-D techniques such as SEM. The 2-D images generated with this mode of AFM image enhancement bears a distinctive similarity to the cellular alignments displayed by the bacteria in the smear monolayer of infected dentinal

tubuli when imaged with the SEM technique (Fig 4). The pocked depressions described in Figure 2 are also a feature of the bacterial topography evident in the scanning electron micrograph illustrated in Figure 4.

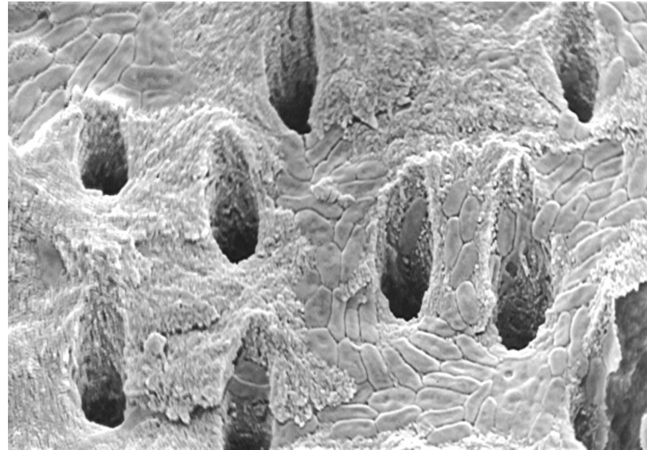


Figure 4. Infected dentinal tubuli of a root canal showing smear layer bacteria arranged in a 'cobblestone paving' pattern (Marais, 2000).

6.5.2 Three Dimensional image manipulation

In addition to the standard top view, the active image can be rotated and displayed from any angle. This image analysis feature permits a more detailed and quantitative evaluation of superficial structures. The depth perspective of the image can be manually adjusted to manipulate the tilt and rotation of the 3D matrix and will change the overall orientation of the image. This feature uses the z scale to emphasise the topography and enhances the relative height of the features in the 3-D image (Zhou and Christie, 1995, Symanski, 2004).

As with other gram positive bacteria, the spherical cell walled *Staphylococcus aureus* bacteria have previously been reported to retain their dimensional proportions as well as cell wall integrity when exposed to biocidal insults (Abid *et al.*, 2004). The same authors have shown that *S. aureus* was more tolerant to the biocidal effects of hypochlorous acid than *E. coli.*, and that extended exposure to low dosages of hypochlorouds acid (2mg/l) resulted in substantial dimensional heterogeneity.

Aside from the topographical clarity of the surface contours displayed in the images of the untreated preparation of *S. aureus* (Fig 5a), the similarity of the characteristic morphological features and dimensions described by other microscopic techniques of lower resolution also validates the integrity of this sample preparation procedure as being substantially artefact free. The relative loss of clarity and the blurred nature of the contours in the Actsol® treated preparation (Fig 5b) are proposed to reflect alterations to the integrity of the cell wall components.

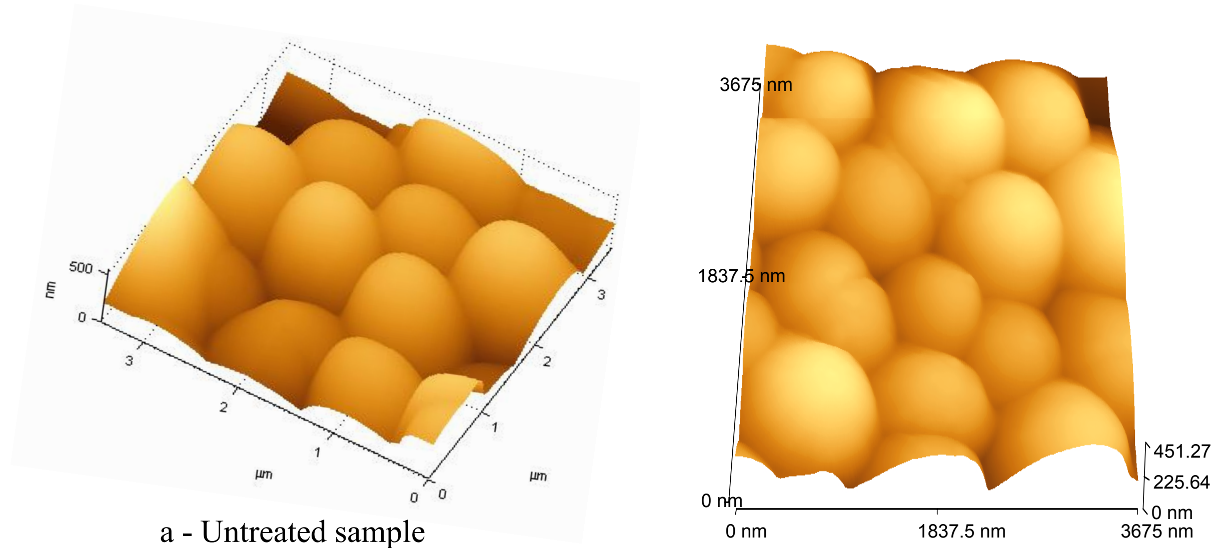


Figure 5. Image enhancement through the tilt and rotational manipulations of the 3-D features of untreated (a) and Actsol® treated (b) *S. aureus* preparations.

Aside from the changes deemed to be associated with biocide exposure, variations in relative cellular dimension are also recognised to reflect the spectrum of different growth stages as manifest by the changes in shape (Doktycz *et al.*, 2003), as well as the surface area to volume ratios which may characterise the normal cell division cycles (Shockman and Höltje, 1994).

6.5.3 Image measurement

The line measure function permits the cross-sectional measurement of a selected image along a user-defined line. By exploiting the x, y, and z matrix data of the image, it is possible to isolate any point along the profile line, and to accurately

determine the point-to-point dimension of the nominated image. By manually manipulating the horizontal and vertical planar options of the software, it is possible to profile any image, and to draw a line at any angle and of any length within the image matrix. In addition to describing the dimensions of the nominated images, it is also possible to map the superficial contours of the sample. This tool is thus complementary to the superficial 2D image scans which rely on colour contrast to describe topographical features as well as the tilt and rotated images which afford enhanced depth of perspective but with limited metrological capacity.

The images detailed in figures 6 and 7 illustrate the features available through the line measurement facility of the Explorer™ software.

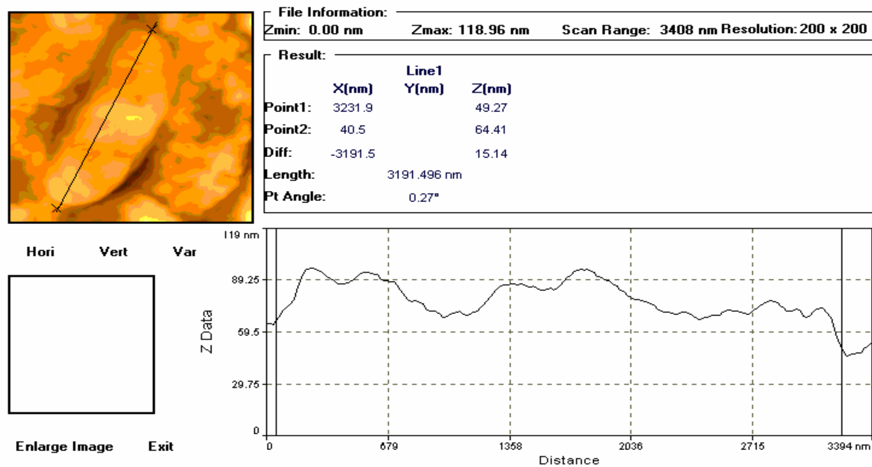


Figure 6. Cross sectional image of the longitudinal topography and dimension of a *P. aeruginosa* bacterial cell after Actsol® treatment.

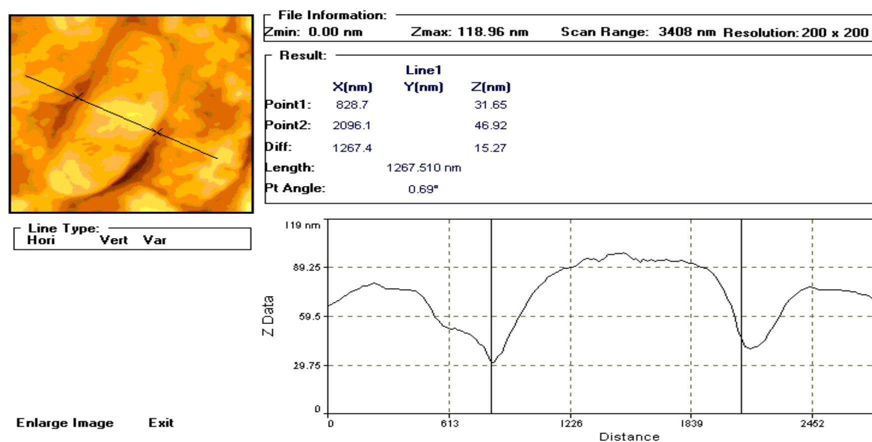


Figure 7. Cross sectional analysis of the transverse topography and dimension of a *P. aeruginosa* bacterial cell after Actsol® treatment.

The irregular surface contour line of the longitudinal section of *P.aeruginosa* detailed in Figure 6 corresponds to the pocked depressions which are described in Figures 2, 3 and 4. Alternatively, the irregular surface contour may also reflect debris associated with leakage of cytoplasmic content but the scope of this study does not permit validation of this proposal.

Measurements of the longitudinal and transverse dimensions of individual bacterial cells were conducted on ten images of each of the untreated control and Actsol® treated preparations. Simple arithmetic means and standard deviations were calculated for each set of data (Table 1).

Table 1. Means and standard deviations of the dimensions of different bacterial strains using line measurements before and after exposure to different Anolyte concentrations (SD- Standard deviation, all measurements in µm)

		Untreated		Actsol® (undiluted)		Actsol® (diluted 1:10)	
Organism	Dimension	Mean	SD	Mean	SD	Mean	SD
B. subtilis	Length	3.8	0.6	3.06	0.61	2.95	0.26
	Breadth	0.96	0.12	0.84	0.10	0.72	0.19
S.aureus	Length	1.34	0.14	1.13	0.14	1.11	0.16
	Breadth	1.19	0.20	1.13	0.09	1.12	0.28
E.coli	Length	2.36	0.19	3.26	0.39	2.7	0.72
	Breadth	0.83	0.13	0.92	0.09	0.89	0.09
P.aeruginosa	Length	2.89	0.50	2.94	0.36	2.49	0.17
	Breadth	1.02	0.28	1.06	0.20	0.86	0.13

While the linear contour tool afforded a real indication of the changes that occurred to the surface topography of the microbes both before and after Actsol® exposure, the comparative integrity of the data from the linear measurement of the Actsol® treated samples was adjudged to be inherently flawed due to the extreme distortions to the cell structure and in many cases, complete lysis of the cell (Table 1).

The exposure of the gram positive bacterial strains to the Actsol® solutions resulted in a reduction in both length and breadth. Given that *S.aureus* is inherently spherical, the

near equivalence of the two perpendicular dimensions confirms the limited structural alterations between the pre-and post-Actsol® exposure images. In converse, exposure of the gram negative strains to the two Actsol® solutions resulted in an increase in both the length and breadth dimensions, and this effect was most obvious with the *E.coli* images. An empirical assessment of the *E.coli* data would suggest that exposure to the undiluted Actsol® solution resulted in a greater dimensional alteration than that displayed by the cells of the same suspension after exposure to the 1:10 Actsol® dilution.

However the extensive range of distortions to the cell dimensions that resulted from exposure to both of the Actsol® solutions would bias any meaningful accordance of statistical significance. While there were minor differences to the changes in dimension between the undiluted and 1:10 dilution samples, it would appear that dilution of the Actsol® to a 1:10 strength, did not result in any dose-dependent or proportionate change in magnitude of cellular distortion and that both solutions resulted in an equivalent amount of cellular damage. It is recognised that under these experimental conditions that further in-depth statistical analysis to determine levels of significance between the pre- and post- Actsol® treatment dimensions would have been spurious. Thus at best, the line measurement tool should be used as a qualitative reflection of the changes in structural dimensions and contours that appear to be causally related to the Actsol® exposure. In order to dilute the inherent bias of the measurements, the length:breadth ratios were calculated and are detailed in table 2.

Table 2. Differences in dimensional ratios between the different bacterial strains before and after exposure to different Actsol® exposure regimens.

	Untreated control	Actsol® (undiluted)	Actsol® (diluted 1:10)
Organism	Length to Breadth ratio		
B. subtilis	3.96	3.64	3.13
S.aureus	1.12	1.03	0.99
E.coli	2.84	3.54	3.07
Ps. aeruginosa	2.83	2.77	2.90

Of particular relevance were the different morphological responses that the various categories of bacteria displayed to Actsol® exposure. While the gram positive organisms revealed minimal dimensional deviations following Actsol® exposure, the membrane bound gram negative bacteria revealed a significant increase in all dimensions which was paralleled by a substantial increase in the presence of extracellular debris following Actsol® treatment.

6.5.4 Image collation

The multiple image manipulations made possible with the AFM software technology provide a composite assessment tool by which to describe the structure and morphological dimensions of microbial populations on a repeatable basis. The images generated either through a superficial 2-D perspective, the 3-D tilt and rotational manipulation or through direct measurement, all provide a unique insight into the changes that exposure to biocidal agents may effect to the integrity of cellular structure relative to the equivalent untreated preparations. Time dependent effects of external agents such as solvents, chemicals and enzymes on the cell surface can be collated over fixed time intervals and can reveal a progressive change in cell surface topography (Dufrene and Müller, 2005). The equivalent time related effects can also be corroborated using Transmission Electron Microscopy (TEM) (Liao *et al*., 2007).

Time-lapse photography of *E.coli* suspensions viewed with AFM reveal that prior to exposure to the oxidant ECA solution, the microbes tended to aggregate in the presence of Extra-cellular Polymeric Substances (EPS). Within 30 seconds of exposure, all cellular dimensions had increased, and after 1 minute, the cellular definition had become obscured due to the overt presence of cytoplasmic material that was associated with membrane rupture and cellular lysis. After 5 minutes, no intact cells could be visualised and the final image comprised of a large amount of cellular debris and lysed cells (Tapper *et al.*, 1998; www.puricore.com, 2007). In a further aspect of the same study, exposure of a suspension of *Desulfovibrio indonensis* to the oxidant ECA solution under equivalent imaging conditions resulted in a similar increase in cellular dimension within 30 minutes of exposure. This coincided with the marked presence of a polar extrudate which was proposed to be associated with the location of the solitary polar flagellum characteristic of this species. After 90 minutes

of exposure, all cells displayed markedly irregular contours and reflected universal cellular lysis (Tapper *et al.*, 1998). In accordance with the latter study which detailed the substantive leakage or extrusion of intracellular contents after oxidant ECA solution exposure, all gram negative preparations as well as that of *B. subtilis*, displayed a widespread presence of extracellular debris after contact with Actsol®. Relative to the equivalent images of the untreated controls, this accumulation of debris was shown to be directly associated with heightened cellular distortion and topographical disruption. This effect is amply evident in the pre- and post-exposure images of *E. coli* illustrated in figures 9 and 10.

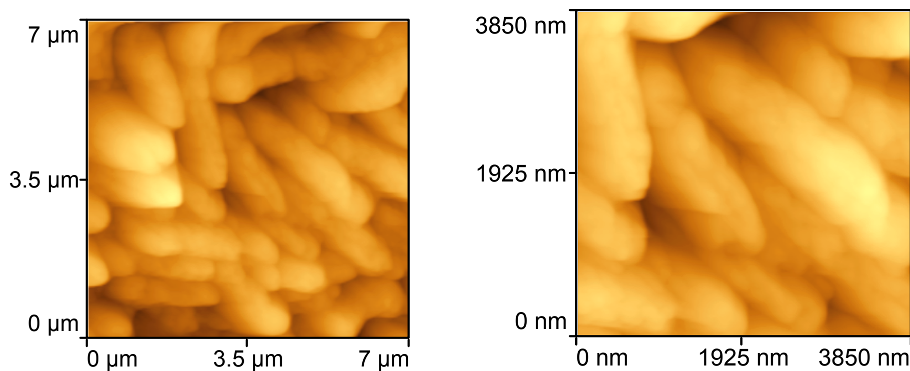


Figure 9. Normal topographic dimensions displayed with enhanced colour contrasted images of an untreated *E.coli* preparation.

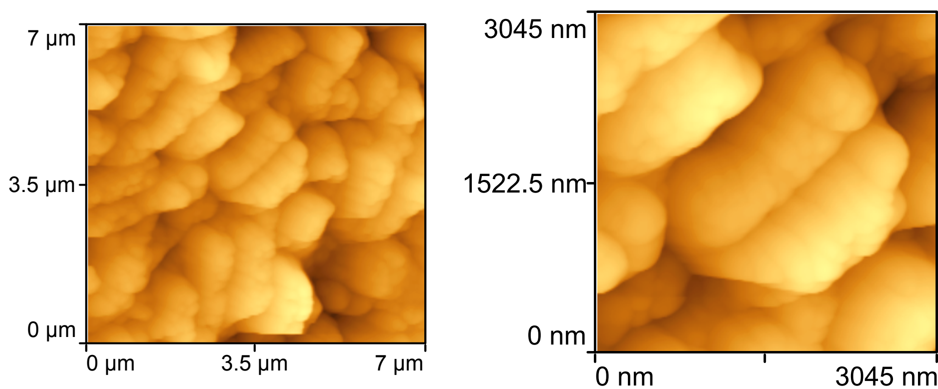
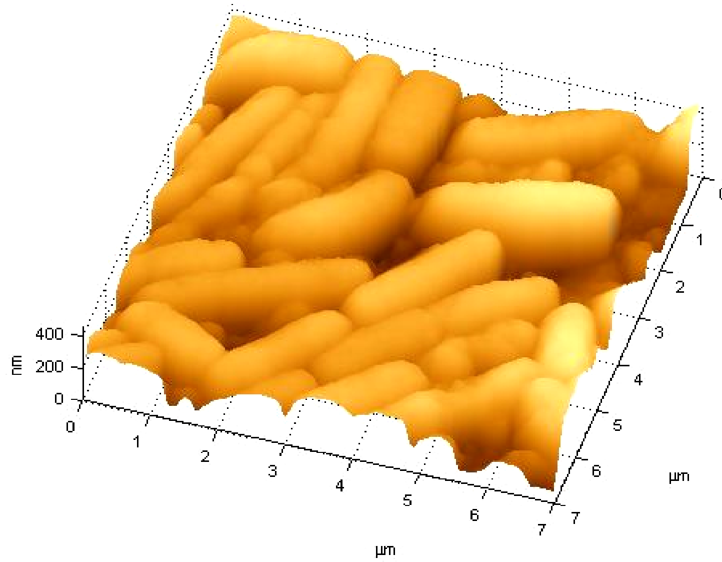


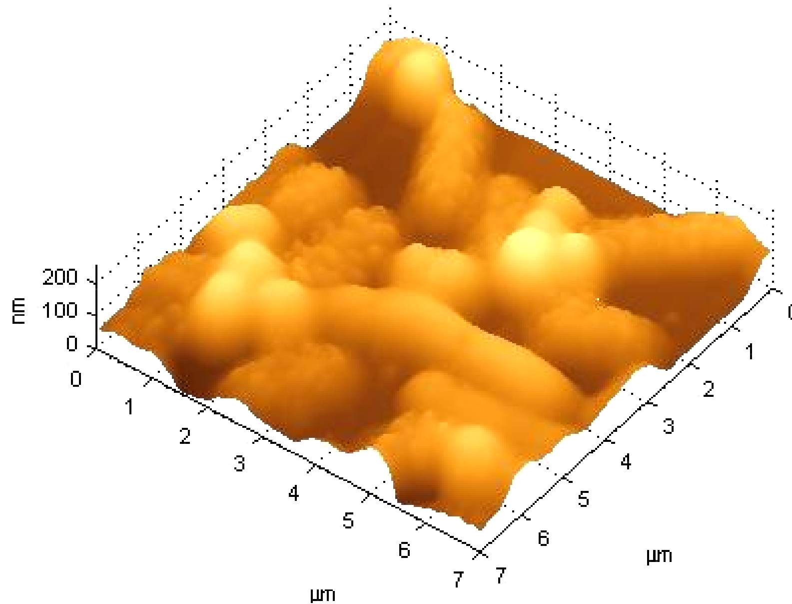
Figure 10. Distinctive swelling and reduction of length displayed with enhanced colour contrasted images of an Actsol® treated *E.coli* preparation

This cellulolytic effect stands in sharp contrast to the post- Actsol® exposure images of *S. aureus* which displayed little or no overt dimensional or topographical changes. In contrast, the rigid cell wall structure of *B. subtilis* afforded the most graphic images of the anolyte mediated cellular disruption and the causally associated accumulation

of extracellular debris (Fig 8b). It is proposed that the extracellular debris present in the image of the untreated *B. subtilis* preparation (Fig 8a) may in part be due to preparation artefacts. Conversely, the distinctly cell associated debris as well as vesicular or 'bleb' type structures on the grossly distorted cells of the Actsol® treated preparation (Fig 8b), would strongly suggest that the affiliated aggregates are a direct consequence of the leakage of intracellular contents.



a) Untreated control



(b) Actsol® treated sample

Figure 8. Differences in 3-D topography between the untreated (a) and Actsol® treated (b) images of *B. subtilis*.

An equivalent finding has been described by Nakajima *et al*. (2004) who reported on a substantive morphological change and bleb formation on the cell wall of a *Pseudomonas aeruginosa* sample that had been exposed to an electrolyzed solution.With progressive exposure they reported that the blebs became elongated and developed a distinctive neck when viewed under TEM. The same 'bleb' effect has been described in *E.coli* using TEM after a 7 second exposure to electrolyzed oxidizing water (Liao *et al*., 2007).

6.6 Conclusions

The use of the AFM technology to image bacterial cells is well established, and despite the limitations of sample preparation and the acknowledged likelihood of image artefacts, it affords a valuable tool to evaluate the effects of exposure to chemical reagents and biocidal compounds in particular. Aside from the capacity to image wet preparations that reflect near physiological conditions, imaging under air offers an array of complimentary software manipulations which consolidate the information derived from single plane images.

Previous AFM studies have revealed that cells swell after a 7-30 second exposure to oxidant ECA solutions and suggest an interference and disruption of the metabolic activity, as well as structural and functional damage to the cell membranes and the wall itself (Tapper *et al*., 1998, Zinkevitch *et al*., 2000, Diao *et al*., 2004; Liao *et al*. 2007).

While the precise mechanism that leads to overall cellular lysis remains unclear, recent evidence would suggest that the effect of reactive oxygen radical species (or Fenton reaction equivalents) on both the cell wall and cytoplasmic barriers initiates a leakage of cyplasmic contents within 7 seconds of exposure of electrolyzed oxidizing solutions in gram negative bacteria (Liao *et al.*, 2007). The final and equivalently explosive lytic effect displayed by cells pre-treated with lysozyme and then placed in a hypotonic solution, would suggest that the massive ingress of water through the compromised barrier structures would play a substantive role therafter (Bolshakova *et al.*, 2001).

Aside from the *S. aureus* preparations, all Actsol® treated bacteria revealed substantial morphometric deviations relative to the untreated controls, and these changes comprised altered geometric contour, collapse and cellular lysis with leakage of intracellular contents. A previous study has reported that *S. aureus* cells undergo limited morphological, yet marked physiological changes when exposed to low doses of hypochlorous acid, but also confirmed that gram positive bacteria were substantially more resistant to hypochlorous acid stress that gram negative strains (Abid *et al.*, 2004).

The similarity of the disruptive changes evidenced in bacterial samples exposed to both chlorine derived and chlorine free anolyte solutions substantiates the hypothesis that reactive molecular electrolytic compounds other than the conventional chlorine based species may play the dominant role in bacterial inactivation (Jeong *et al.*, 2006). This assertion is supported by the substantial attenuation of inactivation of bacteria exposed to electrolyzed oxidizing solutions following the addition of glutathione (Drees *et al*., 2003).

This study suggests the biocidal mechanism of the Electro-chemically activated water solutions to be surface associated and confirms the merits of employing the high-resolution AFM technology to both describe bacterial morphology as well as to evaluate the structural changes that follow biocidal exposure.

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Chapter 7

The efficacy of Electro-chemically Activated (ECA) water against aerosolised *Bacillus subtilis*, *Serratia marcescens*, *Mycobacterium parafortuitum* and *M. tuberculosis* in a controlled environment.

7.1 Introduction

Tuberculosis (TB), one of the world's greatest killers, is predominantly spread by airborne transmission of the pathogen *Mycobacterium tuberculosis*. Infectious droplet nuclei containing *M. tuberculosis* may remain suspended in air for prolonged periods of time, leading to a high risk of infection in congregate settings with poor or little ventilation. Under these conditions, susceptible populations, notably children and immunosuppressed individuals are most at risk of contracting the disease. These high risk environments have been reported to include correctional and health care facilities, public transport systems and schools (Peccia and Hernandez, 2001).

While TB is generally spread through air, inadequately decontaminated medical devices have also been implicated in iatrogenic infections. *M.tuberculosis* can survive for days on inanimate objects and infectious longevity of up to two months has been recorded (Sattar *et al.*, 1995). Iatrogenic infection contracted from contaminated diagnostic bronchoscopes and endoscopes, as well as dental equipment has also been reported (Selkon *et al.*, 1999; Marais and Brözel, 1999; Middleton *et al.*, 2000).

Health care workers in public health facilities in South Africa, a large proportion of whom may be infected with Human Immunodeficency Virus (HIV), are especially vulnerable to nosocomial transmission of *M. tuberculosis*, yet most facilities lack appropriate policies and procedures for infection control. In addition, parallel contamination with *M. avium-intracellulare* has been reported to infect upwards of 50% of Aquired Immunodeficency Syndrome (AIDS) patients (Griffiths *et al.*, 1999). Although infection control in high HIV-prevalence settings is of paramount concern, resource constraints dictate a need for inexpensive administrative and environmental infection control measures. International guidelines to reduce TB transmission in resource-limited settings are available, but although based on sound industrial hygiene

principles, are not supported by scientific data on the effectiveness in the intended settings (World Health Organization, 1999; Centers for Disease Control and Prevention, 2005).

Effective room air disinfectants will have an immediate and significant impact on the transmission of *M. tuberculosis* in congregate settings, representing an inexpensive environmental infection control tool applicable to all resource-constrained settings. Currently, no methodology exists to reliably evaluate the efficacy of aerosolised disinfectants against airborne *M. tuberculosis*, and approaches to date have largely relied upon extrapolations of *in-vitro* data obtained from microbial suspensions exposed to the disinfecting agent.

A unique, state-of-the-art Airborne Infection Research (AIR) Facility, established as a joint partnership between the SA Medical Research Council (SAMRC), the US Centers for Disease Control and Prevention (CDC), the Council for Scientific and Industrial Research (CSIR) and Harvard University in the US, provides a biological model for standardising methodologies for the evaluation of aerosol disinfection. Since *M. tuberculosis* cannot be cultured directly from air, the AIR Facility involves extraction of infectious air from patient wards and supply to animal exposure chambers housing guinea pigs which serve as indirect quantitative samplers of infectious aerosols. The engineering flexibility of the AIR Facility permits experimentation under a variety of environmental conditions (e.g. ventilation rates, temperature and humidity) in addition to optimal ventilation, heating and air conditioning as would be required for compliance with the veterinary ethical standards in South Africa. Guinea pigs are highly susceptible to Mtb infection when air containing infectious droplet nuclei is inhaled, and the probability of infection is proportional to the concentration of infectious droplet nuclei in the air and the volume of air breathed over the duration of the exposure (Wiegeshaus *et al*., 1970). Guinea pig infection is readily determined by tuberculin skin testing after a few weeks of exposure. Effective air disinfection can therefore be indirectly assessed by exposing air laden with TB bacilli to the disinfectant and then measuring the reduction in guinea pig infections over time.

The technology studied in this experiment was based on electrochemically activated (ECA) potable water in a patented reactor system (Bakhir, 1999; Prilutsky and Bakhir, 1997). The mixed oxidant solution generated under prescribed production parameters has demonstrated broad spectrum antimicrobial efficacy and has been trade-marked as Actsol®[1].

Actsol® is a positively charged oxidizing solution comprising hypochlorous acid, hypochorite, hydrogen peroxide, ozone, chlorine-dioxide and chlorine, and has been reported to display potent disinfectant properties (Marais and Brözel, 1999; Selkon *et al.*, 1999; Shetty *et al.*, 1999; Zinkevich *et al.*, 2000). Compared to ordinary liquid disinfectants, the oxidant ECA solutions are regarded as less toxic, less volatile, easier to handle, compatible with other water treatment chemicals and effective against biofilms, while not generating any by-products (Prilutsky and Bakhir, 1997; Middleton *et al.*, 2000).

While the specific mechanisms involved in the inactivation of microbes by the oxidant ECA solutions have not been fully elucidated, the primary reaction appears to involve the oxidation of components of the microbial membrane resulting in an increased permeability, the resultant leakage of macromolecules and ultimately cell death. Recent studies have shown that the main mechanism of inactivation in response to oxidative stress is more subtle and involves the uncoupling of the electron transport chain along with the inactivation of key respiratory enzymes (Helbling and VanBriesen, 2007). Oxidative attack on bacterial proteins may lead to amino acid modification with fragmentation and loss of secondary structure, which results in exposure of hydrophobic residues. These changes favour anomalous aggregation due to hydrophobic interaction and cross-linking reactions (Aertsen and Michiels, 2004).

Actsol® has been reported to be safe in experimental animals, having shown negligible ocular irritation or corrosion potential in rabbits, absence of acute toxicity after oral administration of high doses to rats, and the absence of skin irritation after topical administration in rabbits and pigs (Marais and Brozel, 1999; Marais, 2002).

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[1] Actsol® is the registered trademark of Radical Waters (Pty) Ltd.

Whilst evidence regarding *in vitro* antimicrobial efficacy of the oxidant ECA solution has emerged in the scientific literature (Tanaka *et al.*, 1996; Marais and Brözel 1999; Middleton *et al.*, 2000; Selkon *et al.*, 1999; Shetty *et al.*, 1999; Zinkevitch *et al.*, 2000; Prince *et al.*, 2002), its efficacy as an aerosol or air disinfectant has not been investigated.

7.2 Aims and Objectives

The overall aim of this study was to assess the efficacy of Actsol® in air disinfection of three patient wards challenged with aerosolised *M. tuberculosis* H37Rv, as determined by tuberculin skin testing of guinea pigs exposed to the air from these three patient wards. This was achieved through a systematic, phased approach using three objectives as outlined below.

7.2.1 Phase I

Establishing optimal conditions for and standardisation of Actsol® aerosolisation, bioaerosol generation and bioaerosol collection methodologies. Parameters included relative humidity set to 65% +10%, with approximately 10% of the absolute moisture in the ward air being Actsol® which resulted from a total fogging rate of Actsol® of approximately 2.36 L/h (or 0.787 L/h per patient ward).

7.2.2 Phase II

Establishing parameters for achieving adequate statistical power and demonstrating proof-of-principle that Actsol® had at least 50% efficacy, using three non-TB micro-organisms i.e. *Serratia marcescens, Mycobacterium parafortuitum* and *Bacillus subtilis.*

Bacillus subtilis is a ubiquitous bacterium commonly recovered from water, soil, air, and decomposing plant residue. This bacterium produces endospores that are both temperature and desiccation-resistant in the environment. *Serratia marcescens* has been isolated from eggs of insectaria and are red-pigmented (as opposed to human-isolates of *S. marcescens*) and was non-spore forming. *Mycobacterium parafortuitum*

is an acid fast, relatively fast-growing mycobacterium often used as a surrogate for the slower-growing *M.tuberculosis* in *in-vitro* and *in-vivo* studies.

7.2.3 Phase III

Assessing the efficacy of aerosolised Actsol® in preventing guinea pig infections derived from air contaminated with aerosolised *M. tuberculosis* H37Rv, based on an efficacy of at least 50% reduction in infection rate under defined conditions and within standardised parameters.

7.3 Material and Methods

7.3.1 Preparation of bacterial culture suspensions

Stock cultures of *Bacillus subtilis var niger* (ATCC#49337), *Serratia marcescens* (ATCC#8195) and *Mycobacterium parafortuitum* (ATCC#19686) were procured from the American Type Culture Collection (ATCC) and propagated in accordance with ATCC specifications.

(i) *Bacillus subtilis var niger*

Fresh cultures were prepared from single colonies grown overnight at 37°C in 20ml Bacto trypto soy broth (TSB, Merck, Darmstadt, Germany). A 200ml flask of TSB was inoculated with 1ml *B. subtilis* suspension and incubated for eight days at 37°C in Erlenmeyer flasks in a shaker-incubator. After eight days of growth, a working suspension was prepared by heat-shocking the bacterial broth at 55°C for 30min.

(ii) *Serratia marcescens*

Fresh cultures were prepared from single colonies grown overnight at 37°C in 20ml of nutrient broth (NB, Merck, Darmstadt, Germany). A 200ml flask of NB was inoculated with 1ml *S. marcescens* suspension and incubated overnight at 37°C with vigorous shaking.

(iii) *Mycobacterium parafortuitum*

Fresh cultures were prepared from single colonies grown for 72hrs at 37°C on Bacto tryptic soy agar plates (TSA, Scharlau Chemie SA, Barcelona, Spain). Fresh cultures were scraped from agar plates and suspended in sterile phosphate-buffered dilution water (PBDW). To check for contamination, colonies were plated on blood agar plates, incubated for 24hrs at 37°C and stained with Ziehl-Neelsen (ZN) before examination with microscopy to confirm acid fastness. Immediately prior to each experiment, bacterial cultures were aseptically transferred to 50ml sterile conical centrifuge tubes (Falcon® 2076, Becton Dickinson, SA), and centrifuged at 2 500g for 20min using an ALC Centrifuge (PK130). The supernatant was discarded and the pellets were re-suspended in sterile PBDW. The washing process was repeated twice more and the cells re-suspended a final time in 100ml of sterile PBDW.

Prior to aerosolisation, cell concentrations of the washed bacterial suspensions were determined by turbimetric measurement using a Beckman DU-65 spectrophotometer (Irvine, California, USA). The concentration of bacterial cells in the suspension was estimated from previously developed titration curves comparing turbidity of the suspension with colony-forming units (CFU). A dilution series of the washed cell suspension was also plated to determine the original concentration. The washed cell suspension was diluted to prepare 100 ml of nebuliser suspension at concentrations ranging between 10^5 and 10^8 cells per ml. Before and after nebulisation, a dilution series was performed for standard plate count enumeration at high and low bacterial concentrations. Plates were incubated at 37°C for 24h (*S. marcescens, B subtilis*) and 48-72h (*M. parafortuitum*) for enumeration of viable bacteria.

7.3.2 Aerosolisation and air sampling of bacteria

A six-jet modified MRE-type Collision nebuliser was used to aerosolise the suspensions of bacterial cells in the three patient wards of the AIR Facility. The combined air volume of the three patient wards was $112.06m^3$.

Andersen six-stage samplers (ThermoElectron, Smyrna, Georgia, USA) were used to sample the air at quasi-random positions in the animal chambers. The flow rate through the Andersen six-stage sampler was adjusted to 28.3 L/min. Sampling plates

were prepared by aseptically pouring 45ml of the appropriate medium into each of the six 100mm x 15mm sterile plastic Petri dishes (model 0875713, Fisher Scientific, Atlanta, Georgia, USA) so that the gap between the nozzles and agar surface met the manufacturer's specifications. All inside surfaces were maintained in sterile conditions until sampling. The samplers were operated for periods of 1min and 10min sampling time, according to the specific micro-organism, the airborne concentration, and whether or not Actsol® was being fogged. The Andersen six-stage samplers operated at a nominal airflow rate of 28.3 L/m; thus, sampling for 1min or 10min resulted in a total sample volume of approximately 28.3 or 283L of air. After sampling for the prescribed period of time, the samplers were removed from the animal rooms. Next, the plates were removed from the samplers, covered, inverted and incubated at 37°C and enumerated after 24hrs (*S. marcescens, B. subtilis*) or 48-72hrs (*M. parafortuitum*). The CFU counts on all six plates from each Andersen six-stage sampler were combined for analysis.

7.3.3 Animal husbandry

Specified pathogen-free Dunkin Hartley guinea pigs (male and female) weighing between 250g and 300g were procured from South African Vaccine Producers, Sandringham, Johannesburg. Guinea pigs were paired at birth and transported to the AIR Facility under sterile conditions in Techniplast cages equipped with 0.22 μm filters under a stable temperature of 22 ± 4°C. Guinea pigs were delivered within 4 hours after dispatch. Upon arrival at the AIR Facility, guinea pigs were transferred to wire cages at predetermined random rack positions, each coded from left to right and from top to bottom, resulting in a unique identification number for each cage and each animal.

Based on an assumed cumulative infection rate of 80% in the unexposed group of guinea pigs and a doubling of the sample size to accommodate any potential variation resulting from randomisation, a total of 74 animals were housed in pairs in each of the two animal rooms. Six additional guinea pigs were placed at random in three cages in each of the rooms and sacrificed after one week to validate that the animal exposure

chambers were free from contamination and that the animals did not suffer from stress-related conditions.

After allowing the animals to acclimatise for two days, guinea pigs were individually marked and weighed. Baseline tuberculin skin testing was done by intradermal injection of 100TU/0.1ml protein purified derivate (PPD), (Mycos Laboratories, USA). PPD was prepared immediately prior to testing and administered on a depilated area of the back of each guinea pig. No delayed-type hypersensitivity reactions were observed, confirming the absence of any TB infection prior to the exposure. Guinea pigs were maintained under biosafety level 3 conditions at constant temperature of 22°C ± 4°C, relative humidity of 55% ± 10% and day/night cycles of 12 hours. The combined air volume of the two animal exposure rooms was 24.43m^3.

Trained animal care takers were responsible for daily care of the guinea pigs under protocol conditions (daily provision of sterile, de-ionised drinking water augmented with water-soluble ascorbic acid at a concentration of 3mg/ml, autoclaved rabbit pellets, autoclaved *Eragrostis* hay and vermiculate chips in waste collection trays. Cleaning of waste collection trays and the animal rooms occurred three times per week.

7.3.4 Environmental parameters

Internal environmental conditions for the AIR Facility patient wards (six air changes per hour) and animal exposure chambers were pre-set according to protocol specifications to ensure maximum transfer of both aerosolized *M. tuberculosis* H37Rv and Actsol® from the patient wards via the exhaust air transfer duct system to the animal exposure chambers.

Patient wards were kept under negative pressure, with the windows permanently sealed and the doors closed. Trend data for temperature, humidity, air changes per hour, filter pressure differentials and alarms were monitored electronically to detect deviations from the pre-set norms.

7.3.5 Preparation of Actsol®

The electrochemically activated oxidant solution (Actsol®) was produced by a commercial electrolytic generator system at the premises of Radical Waters, (Kyalami, South Africa). The device was configured and preset to electroactivate a dilute brine solution comprising 2.5 gm/litre food grade Sodium Chloride (NaCl) of 98% purity, and the production parameters of the Actsol® solution were recorded (Table 1).

Table 1 Actsol® solution specifications

Parameter	Value
Oxidation-Reduction Potential	> 950 mVolts
pH	6.7±0.2
Electrical Conductivity:	5.0 ± 0.5 mSiemens/cm
Free Available chlorine	180 ± 20 mg per litre

In order to detail any potential variance between the volume of deionised water used as the fogging control relative to that of the Actsol® solution, the specific gravity of the Actsol® solution was measured and recorded as being 0.9555 (Food Consulting Services, 2006).

In addition, a sample of the Actsol® solution was submitted to an independent laboratory for physical and spectrophotometric analysis to confirm the composition of the solution supplied for fogging (Table 2).

Table 2. Results of the physical and spectrophotometric analysis of the Actsol® solution (Claassens, 2006).

Parameter	pH	EG mS/m	Ca mg/L	Mg mg/L	K mg/L	Na mg/L	S mg/L	Cl mg/L	HCO mg/L	NO_3 mg/L
Value	6.7	349	4	3	2.1	720	30	1873	83	11

7.3.6 Aerosolisation of Actsol®

An AQUAFOG TurboXE-1500 (Jaybird Mfg. Inc., PA, USA) centrifugal atomiser fan was used during this study. Continuous flow of Actsol® solution was maintained with an independently calibrated centrifugal pump (Grundfos DME IP65, 18W) preset to deliver 40 ml/minute. At this flow rate, the manufacturer advised that the average droplet diameter would be ≤ 5µm and that less than 3% of droplets would be in the 5-50 µm range. Determination of the ranges of droplet sizes during the atomiser calibration phase confirmed that droplet diameters were progressively reduced in size as atomisation proceded and that a steady state with >95% of droplets being recorded in the <10 µm range was attained after 40 minutes of atomisation.

Under the preset environmental conditions (temperature and RH), it was confirmed that the majority of atomised droplets would have evaporated shortly after aerosolisation. Further control of the solution delivery rate was achieved by mounting the container with the Actsol® solution on a calibrated scale (Vibra AJ 62005, Calibration Certificate 232521) and recording the weight over 5 minute intervals for the duration of the fogging period.

Finally, overall Actsol® usage was confirmed by both internal and independent measurement of the weight of the designated containers before and after the fogging intervention. Actsol® was aerosolised in all of the three patient wards, according to protocol procedure. The generator aerosolising the Actsol® was positioned at the face of the supply air grille to each ward. The concentration of Actsol® in the extracted air volumes was estimated to be 10% of the final absolute humidity as recommended by the manufacturer. At six Air Changes per Hour (ACH), 99.9% clearance of Actsol® was calculated to occur at 69 minutes after termination of the aerosolisation of Actsol® in the patient wards. Log sheets of Actsol® aerosolisation were kept independently by on-site representatives of the manufacturer, who were blinded as to the experimental procedures in order to preserve the scientific integrity of each experiment.

7.3.7 Preparation of *M. tuberculosis* H37Rv suspensions

M. tuberculosis H37Rv (ATCC #27294) was cultured in Dubos broth (Sigma, Steinheim, Germany) containing 5% glycerol (Calbiochem, Darmstadt, Germany) and 0.025% Tween 80 (Merck, Munich, Germany) at 37°C for three weeks, and gently vortexed daily to minimise clumping. The cells were subjected to centrifugation at 1500g for 20 minutes, followed by re-suspension in sterile phosphate-buffered saline (PBS), and this procedure was repeated twice.

The concentration of the washed cell suspension was estimated by turbimetric comparison with McFarland No.1 standard as well as a turbidity measurement at OD 600 of the McFarland No.1-equivalent cell suspension. The McFarland No.1-equivalent cell suspension of *M. tuberculosis* H37Rv was estimated to contain 10^9 CFU/ml. Eighteen 100ml nebulisation suspensions with a target concentration of $5x10^2$ CFU of *M. tuberculosis* H37Rv/ml were required per day of exposure; therefore 36 x 10ml aliquots were prepared from the stock cell suspension to cover both exposure days.

Immediately prior to nebulisation, one aliquot was added to 90ml of PBDW and used as the nebulising suspension. Undiluted and 10-fold dilutions of each nebulisation suspension as well as selected dilutions of a titration series of McFarland No. 1-equivalent cell suspension were plated out in triplicate on Middlebrook 7H10 agar plates supplemented with 10% Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment medium (Becton Dickinson, Le Pont de Claix, France) and incubated at 37°C for four weeks. A stock suspension of 5×10^5 CFU *M. tuberculosis* H37Rv /ml in PBDW was simultaneously prepared and frozen in 1ml aliquots with the remaining original stock solution (prepared from broth culture) at -80°C in the event of a need for repeat exposure.

7.3.8 Experimental process

On Day 1 of Phase 3, guinea pigs in Animal Room 2 were exposed to air containing aerosolised *M. tuberculosis* H37Rv at a target concentration of 5×10^2 CFU/ml, and concurrently treated with fogged water (as control solution to Actsol®). Eighteen

100ml *M. tuberculosis* H37Rv nebulisation suspensions were used, i.e. six suspensions per each of the three patient wards, generated over six hours in six 20-minute cycles. On Day 2 of Phase 3, guinea pigs in Animal Room 1 were exposed to air containing aerosolised *M. tuberculosis* H37Rv at a target concentration of 5 x 10^2 CFU/ml, concurrently treated with aerosolised Actsol®. Eighteen 100ml *M. tuberculosis* H37Rv nebulisation suspensions were used, i.e. six suspensions per each of the three patient wards, generated over six hours in six 20-minute cycles.

7.3.9 Guinea pig health surveillance

Guinea pigs were subsequently subjected to weekly health surveillance during which mass measurements were taken and animals were observed for clinical signs and symptoms of ill health following standard protocol procedures.

Three weeks after the initial exposure, an ascorbic acid deficiency was detected in the guinea pigs. This was reversed by means of subcutaneous administration of a dose of 1ml 250mg/ml ascorbic acid to each guinea pig (as advised by the consulting veterinarian) and by increasing the amount of ascorbic acid in the drinking water to 4mg/ml. The ascorbic acid deficiency was deemed to have been corrected after one week of treatment, and was based on full recovery of all guinea pigs with respect to mass gain, food and water consumption and absence of painful locomotion. As a precautionary measure and on the advice of the consulting veterinarian, the second round of tuberculin skin testing was delayed until seven weeks post-exposure to overcome any possible adverse effect of immunological anergy.

7.3.10 Tuberculin skin testing

Tuberculin skin testing (TST) at seven weeks post-exposure was performed following the identical methodology to that of the baseline testing described earlier, i.e. intradermal injection of 100TU/0.1ml protein purified derivate (PPD), (Mycos Laboratories, USA).

PPD was prepared immediately prior to testing and administered to a depilated area of the back of each guinea pig. The assessment of skin test indurations was performed

using digital calipers (Wilson Wolpert, The Netherlands). Two independent readings were taken at right angles to each other (length and width) at 24hrs post-administration, and the results recorded separately in millimetres. The final induration size was calculated as the average from these two readings. Guinea pigs with positive TST reactions were sacrificed in accordance with protocol procedure.

7.3.11 Repeat exposure

Protocol specifications indicated a requirement for repeat exposure of those animals that had not converted their tuberculin skin tests (see Results section). An identical experimental procedure to the first exposure was followed, utilising the frozen aliquots of *M. tuberculosis* H37Rv suspensions as described earlier.

Dilutions of both the stock solution as well as the nebulising solutions were plated in triplicate on selective Middlebrook 7H10 agar plates supplemented with 10% OADC enrichment (Becton Dickinson, Le Pont de Claix, France), incubated at 37°C for four weeks and enumerated. TST was repeated at seven weeks post (re)-exposure, employing identical methodology as before.

7.4 Results

Table 1 summarises the data from Phase I, demonstrating a marked and statistically significant antimicrobial effect ($p<0.001$) of Actsol® at equilibrium concentration (approximately 10% of the absolute humidity) on the number concentration of the test aerosols, i.e. mostly single endospores of *B. subtilis*, and mostly single vegetative cells of *M. parafortuitum* and *S. marcescens* at two concentration titres (high and low). The reduction of contaminated aerosols of culturable air particles was 91-92% for endospores of *B. subtilis*, 63-67% for *M. parafortuitum*, and 86-89% for *S. marcescens* for the high and low titre challenges respectively.

Detailed results for Phase I also revealed a tendency for an agglomeration clumping of particles when the Anolyte® was fogged, with the size distribution of particles shifting to the right (i.e., larger air particles were collected) in the six-stage samplers, at both high and low titres of the respective aerosols.

Table 1. Summary statistics for equilibrium airborne concentration with and without Actsol®, at two titres of test aerosols.

Microorganism	Airborne concentration Actsol® OFF		Airborne concentration Actsol® ON	
	Geometric mean	95% Confidence interval	Geometric mean	95% Confidence interval
	CFU/m^3	CFU/m^3	CFU/m^3	CFU/m^3
B. subtilis				
Low titre	3 416	2 888 – 4 041	180	119 – 272
High Titre	10 794	8 626 – 13 506	500	379 – 659
M. parafortuitum				
Low titre	36 484	29 322 – 45 396	11 939	8 933 – 15 955
High titre	102 912	58 871 – 179 897	38 456	34 428 – 42955
S. marcescens				
Low titre	189	130 – 275	27	20 – 36
High titre	1 264	790 – 2 019	144	75 – 275

Phase II data are summarized in Table 2, where high-titre concentrations of the respective organisms were aerosolised. Phase II confirmed the proof-of-concept that aerosolized Actsol® at equilibrium concentration (approximately 10% of the absolute humidity) significantly reduced the concentration of air borne *M. parafortuitum*, *B. subtilis* and *S. marcescens* counts by at least 50% ($p<0.001$). Results for phase II also indicate that it took approximately 40 minutes for the Actsol® concentration to reach equilibrium and for the maximum inactivation of the three respective microbial aerosols.

Table 2. Summary statistics for reduction in airborne concentration, Phase II.

	% Reduction in aerosol concentration at three time points		
Microorganism	1st Sampling period (10 minutes)	2nd Sampling period (10 minutes)	3rd Sampling period (10 minutes)
	%	%	%
B. subtilis	-33	-44	-90
M. parafortuitum	-71	-92	-95
S. marcescens	-65	-71	-99

Analyses of the AIR Facility system data confirmed no statistically significant differences in temperature, humidity or air changes per hour between the two animal rooms during the course of the experiment, and confirmed that the AIR Facility functioned according to the pre-set criteria outlined in the study protocol.

Phase III data are summarized in Tables 3 and 4, and indicate that Actsol® did not show a statistically significant bactericidal effect on aerosolised *M. tuberculosis* H37Rv under the controlled conditions identified in Phase I and Phase II of the study. On the first exposure, 39.2% of control animals and 42.5% of Actsol® intervention animals converted their respective TST tests (indicating TB infection), while 72.7% and 61.7% respectively converted their TST test after the second exposure.

In total, 83.6% TST conversions occurred in the control animals and 78.1% conversions in the intervention animals, confirming that the study aim of a cumulative infection rate of around 80% in the control arm was achieved and that both the concentration of *M. tuberculosis* H37Rv as well as the animal sample size were adequate to detect any possible differences between the two animal rooms

Statistical analyses using the Pearson chi-squared, Fisher's exact, and 1-sided Fisher's exact tests confirmed the limited antimicrobial efficacy of Actsol® on airborne transmission of *M. tuberculosis* H37Rv (Table 4).

Table 3. Guinea pig tuberculin skin test results, Phase III.

Exposure	Intervention (with fogged Actsol®)			Control (with fogged water)		
	TST +	TST-	Total	TST+	TST -	Total
First exposure	31	42	73	29	45	74
Second exposure	26	16	42	32	12	44
Combined data	57	16	73	61	12	73*

(*Note:1 guinea pig in the Control animal room 2 died between first and second exposure; TB was confirmed by histopathology) TST – Tuberculin Skin Test.

Table 4. Statistical tests for differences in infection rates between the animal rooms.

Exposure	Pearson chi-square	Fisher's exact	1-sided Fisher's exact
First exposure	0.1633 Pr = 0.686	0.739	0.407
Second exposure	1.1462 Pr = 0.284	0.359	0.200
Combined data	0.7070 Pr = 0.400	0.529	0.264

7.5 Discussion

The enhanced risk of nosocomial and iatrogenic spread of mycobacteria, especially drug-resistant strains of *M. tuberculosis*, is forcing a critical review of infection control in general and the claims of mycobactericidal activity of disinfectants in particular.

Mycobacteria are generally more resistant to in-vitro disinfection than enveloped viruses and other strains of vegetative bacteria; however, conventional *in- vitro* testing of disinfectant efficacy suffers from several methodological flaws. These include (i) a lack of proper quantification; (ii) unrealistically long contact times at higher than ambient temperatures; (iii) absence of a suitable organic load; (iv) ineffective neutralizers; (v) unsuitable surrogates for *M. tuberculosis*; (vi) improper recovery media; and (vii) inappropriate types of carriers. These many considerations make the available data on biocide product efficacy against TB unreliable.

Traditional fumigation of contaminated airspaces using conventional biocides and formaldehyde in particular has largely been discontinued due to its long exposure requirements, mycobacterial resistance, hazardous nature, irritancy to eyes, skin and respiratory mucosa and its role as a human carcinogen (Ayliffe *et al*., 1992; Griffiths *et al*., 1999; Middleton *et al*., 2000; Chapman, 2003; Johnston *et al*., 2005).

The kinetics of the disinfectant inactivation of the non-tuberculous *M.avium* has been reported to be two phased and it is characterised by an initial rapid inactivation stage coupled to a slower second stage which describes pseudo-first order kinetics (Luh and Mariñas, 2007). The $CT_{99.9\%}$ (Concentration [mg/L] x Time [minutes] for a $3Log_{10}$

reduction in microbial count) of the chlorine compounds for the *M. avium* strains was shown to be up to 2,300 times that which was calculated for *E. coli* under equivalent test conditions (Taylor *et al*., 2000).

The inconsistent efficacy of disinfectants against different Mycobacterial strains (Rikimaru *et al*. 2000) as well as the inappropriate choice of less resistant mycobacterial strains as surrogate indicators of *M.tuberculosis* susceptibility, exacerbates the difficulty of quantifying consistently reliable disinfectant methodologies against these critical disease causing microbes (Best *et al*. 1990; Sattar *et al*. 1995). It has been reported that the physiological profile of the strain plays a substantial role wherein the slow growing *Mycobacterium avium* was shown to be 10 fold more resistant to oxidant disinfectants than other faster growing Mycobacterial strains (Chapman, 2003). It been shown that disinfectants with general biocidal efficacy against most other microorganisms are not necessarily effective against *M. tuberculosis* and that the tuberculocidal activity of disinfectants varies greatly depending on the mycobacterial species assessed (Best *et al*., 1990; Sattar *et al*., 1995; Griffiths *et al*., 1999; Rikimaru *et al*., 2000).

To date, there has been a limited number studies reporting on the use of atomised electrolysed oxidant solutions for airspace decontamination. Wu *et al*., (2008) describe a 4 log_{10} reduction in *B. subtilis* spores after 45 minutes of ultrasonic nebulisation of dental artefacts in an enclosed and static airspace with acidic (pH 2.5) electrolysed oxidant solution The chlorine based oxidant concentration applied over the entire exposure period equated to a total Free Available Chlorine (FAC) dose of 90,000 mg. In a similar report, anolyte at a pH of 5.2 was used to determine the antimicrobial efficacy of a fog treatment against methicillin-resistant *Staphylococcus aureus* (MRSA) and *Acinetobacter baumannii* inoculated onto ceramic tiles within an enclosed airspace. A single fogging treatment was reported to result in a 10^4 fold reduction for MRSA and a $10^{5.8}$ fold reduction for *A. baumannii*. The output of the fogging system employed was 19 liter per minute, and with an oxidant FAC concentration of 180 mg/L, this equated to 34,200mg FAC delivered into a static airspace during the 10 minute exposure period (Clark *et al*., 2006).

In accordance with the extensive range of electrolytic reactor systems available, there is an equally diverse array of oxidant solution types that can be appraised (Bakhir, 1999; Gauw *et al.*, 1999; Selkon *et al.*, 1999; Shetty *et al.*, 1999; Nakae and Inaba, 2000; Sampson and Muir, 2002). Variations in solution pH are acknowledged to reflect significant differences in the predominant reactive chlorine species in the solution. In a free chlorine inactivation study of *Mycobacterium avium,* it was reported that at a pH of 6, the primary inactivation was due to hypochlorous acid and that the biocidal contribution of the hypochlorite ion was insignificant (Luh and Mariñas, 2007). Highly acidic anolyte solutions (pH<3) are known to comprise of mainly aqueous chlorine, neutral anolytes comprise predominantly of hypochlorous acid, while alkaline anolytes are near exclusively hypochorite ion based (White, 1992; Bakhir *et al.*, 2003).

An initial screening study of the inactivation efficacy of a 10% (v:v) strength Actsol® solution (pH 6) against a 6 $\log_{10}$ suspension of *M. smegmatis*, revealed a 99.7% kill rate after 10 minutes, while a 1 minute exposure to a 90% (v:v) strength anolyte solution, resulted in a >99.9 inactivation of the original titre. Similarly, when a 6 $\log_{10}$ challenge of a washed clinical isolate of *M. tuberculosis* suspension was exposed to a 50% (v:v) anolyte solution, 99.9% inactivation was achieved after 5 minutes. An equivalent exposure duration to a 90% (v:v) anolyte concentration resulted in a total inactivation of the original challenge. However exposure of an unwashed expectorated sputum sample containing 6 $\log_{10}$ *M. tuberculosis* organisms did nor display any inactivation after a 15 minutes exposure to a 50% (v:v) strength anolyte solution (van Zyl *et al.*, 2001). This finding confirms the antibiocidal nature of sputum as detailed in an earlier report (Middleton, *et al*, 2000).

In addition, this finding confirms the contention that tubercule bacilli in sputum display a greater resistance to disinfectant activity, and it is proposed that the active biocidal agent needs to penetrate through the surrounding organic matter without becoming neutralised prior to exerting any inactivating effect (Best *et al.*, 1990; Sattar *et al.*, 1995; Cloete, 2003). In-vitro studies using poloxamer gel constructs to mimic the extra-cellular polysaccharide capacity of microbes to neutralise biocidal agents, substantially confirms the neutralising effect that sputum or an equivalent

extracellular organic matrix would exert upon a targeted biocidal intervention (Wirtanen *et al,*. 1998).

The unique mycolic acid based cell wall structure of Mycobacteria is also deemed to play a pivotal role in determining the specific chlorine demand required for microbial inactivation. In support of this assertion, Helbling and VanBriesen (2007), report that the chlorine contact time for a 3 log inactivation of *Escherichia coli, Staphylococcus epidemidis* and *Mycobacterium aurum* was 0.032±0.009, 0.221±0.08 and 42.9±2.71 mg min/L respectively.

Phases I and II of this study indicated that Actsol® when fogged according to the protocol specifications, has the potential to reduce the number concentration (cfu/m^3) of *B. subtilis, M. parafortuitum*, and *S. marcescens* by at least 90%. Thus fogged Actsol® displays distinctive promise for its ability in killing, inactivating or agglomerating aerosols of these three organisms, and indicates a potential for selective air disinfection in non-TB settings after continuous exposure for at least 40 minutes. Results from this study confirm previous observations (Rikimaru *et al.,* 2000) from conventional *in-vitro* testing of disinfectants, indicating that *M. tuberculosis* is more resistant to conventional biocides than other bacteria. By extrapolating from the antimicrobial efficacy data generated from the direct sampling of aerosolised microbial strains after Actsol® exposure, a presumptive model was extended to the indirect assessment of the capacity of fogged Actsol® to reduce the airborne transmission of *M. tuberculosis.* However, the design of the current protocol did not achieve a statistically significant reduction in infection rates in exposed guinea pigs and therefore should not be considered as an appropriate room air disinfectant in TB settings under the specific application parameters as outlined above.

Phase II of the study enabled the refinement of the optimal target concentration of *M. tuberculosis* H37Rv to be used for assessing infection rates in the experimental animal component of the study. This phase confirmed that the exposure concentration was appropriate for the achievement of an infection rate of just over 80% in the control animals. In the absence of an alternative methodology to culture *M. tuberculosis* from the air, a biological approach such as the AIR Facility model may contribute to the study of the biocidal potential of disinfectants to reduce or eliminate airborne TB

infection in health care settings. The use of standardised protocols to assess *in-vivo* efficacy of disinfectants should thus make evaluation of such agents more precise and reliable.

Further to the findings of the phase II data, it was established that a 40 minute lag period was the minimum time period that was required prior to the attainment of Actsol® equilibrium in the aerosolised airspace. This equates to the minimum time period that would be needed before the minimum inhibitory concentration of aerosolised Actsol® required for optimal (>90%) airborne microbial inactivation would be achieved. From a theoretical perspective, the prescribed nebulisation rate (100ml of 5 x 10^2 CFU/ml over 20 minutes), would equate to 7.5 x 10^3 aerosolised Mtb organisms being extracted from the three patient wards within the first 10 minute period (equal to 1 ACH). Over the same 10 minute period, the amount of Actsol® dosed into the system would have been 400ml. At a Free Available Chlorine (FAC) concentration of 180mg/L, this equates to a total of 72mg FAC. When diluted throughout the three patient ward air volumes of 112m^3 (=1 ACH volume), this equates to 0.64mg FAC/m^3. The same airspace would theoretically contain 670 CFU/m^3 aerosolised Mtb organisms and would have been supplied to the 12.2 m^3 of airspace housing the susceptible guinea pigs.

It is also likely that the previously reported FAC attenuating effects exerted through increased chlorine demand by specifically mycobacterial species (Helbling and VanBriesen, 2007), as well as the substantive reduction of FAC that follows inappropriate atomisation of the anolyte solution (Hsu *et al.*, 2004), would have further reduced the concentration of free chlorine available that would have been required to effect any mycobacterial inactivation. The latter study reported up to 97% reduction of FAC concentration and oxidation reduction potential of electrolysed oxidant solutions that had been atomised through small orifice nozzles, and it was proposed that the reduced volume to surface area ratio of the atomised droplet would substantively contribute to the increased volatilisation of chlorine and hence loss of mycobactericidal activity under these conditions.

While the minimum infectious dose of aerosolised *M. tuberculosis* organisms required to produce a positive TST reaction was not been determined during this study, it has

been reported that a single viable organism may be all that is necessary to result in patent disease (Twang *et al*., 2006).

Thus it thus feasible that a sufficient dose of infectious aerosolised Mtb microorganisms would have been extracted from the patient wards during the initial 10 minutes of Actsol® application. This initial air volume would have been extracted well before the projected time by which the Actsol® MIC equilibrium would have been achieved. This initial ACH would have contained a theoretical dose of 7.5×10^3 potentially infectious CFU and would have been supplied directly to the susceptible animals in the exposure room. To this end, it is possible that substantially improved inactivation statistics for *M. tuberculosis* H37Rv may have been achieved had the patient wards been pre-conditioned with the atomised anolyte solution for a minimum period of 40 minutes prior to the aerosolisation of the challenge microorganism. This contention is supported by the reported need to apply a pre-conditioning period where UV irradiation was assessed against airborne microbial challenge (Griffiths *et al*., 2005).

While no definitive delivery system for the aerosol application of Actsol® or directly equivalent solutions has been reported, the associates of the inventor (Bakhir) have patented a delivery system that is purported to address the engineering shortfalls of previously ineffective atomisation attempts (Tsikoridze *et al*., 2000).

7.6 Conclusions

Based on the findings of this study, the proposed Actsol® application protocol and its specifically prescribed application parameters should not be recommended for air disinfection in TB settings. Nevertheless, the non-toxic nature of Actsol®, together with the reduction in the viable counts of selected bioaerosols demonstrated in the study, justify further investigation.

Based on the findings of the study, it is clear that biocidal interventions to address environmental decontamination requires a holistic understanding of both the manifold biological aspects of the intervention strategy as well as the mechanical or engineering constraints that the intervention may entail.

On a more fundamental level, issues that should receive additional attention include the mechanisms leading to the change in particle size distribution, the ideal bactericidal composition of Actsol® solution, the optimal bactericidal concentration (mg/m^3) of Actsol® applied to the airspace, the ideal particle characteristics for maximum bactericidal action, as well as the design of the equipment to maximize Anolyte aerosolisation.

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Chapter 8

Application of ECA solutions to control nosocomial infections in a Neonatal Intensive Care Unit

8.1 Abstract

In response to frequent outbreaks of *Klebsiella spp* infection in the Neonatal Intensive Care Unit (NICU) in a large referral hospital in Botswana, it was proposed that the existing infection control strategy be augmented with the inclusion of an Electro-Chemically Activated (ECA) oxidant water solution - Actsol®[1], for the disinfection of equipment and contact surfaces within the facility.

The study was carried out primarily in the Neonatal and Postnatal wards and was later extended to include both the male and female surgical wards. The results confirm that disinfection with the Actsol® solution significantly reduced the incidence of all microorganisms on designated surfaces. Overall, a 57 fold reduction in total viable bacterial count was recorded on all surfaces cleaned with Actsol® solution, while surfaces cleaned according to existing procedures with conventional chemicals only displayed a 7 fold reduction.

The incidence of new *Klebseilla spp*. infections in the NICU was eliminated within one week of the Actsol® intervention and the sources of previously persistent *Klebsiella spp* contamination was eliminated. It appears that the continuous availability of the Actsol® solution in this confined environment was effective in controlling pathogen transmission.

The study confirms that the use of Actsol® can assist in the control of *Klebsiella spp.* outbreaks in neonatal wards, and could be used to enhance the overall hygiene conditions within other hospital wards.

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[1] Actsol® is the registered trade mark of Radical Waters

8.2 Introduction

Nosocomial or Hospital Acquired Infections (HAI) are one of the leading causes of morbidity and mortality in Neonatal Intensive Care Units (Borghesi and Stronati, 2008). Outside of the obvious requirement for optimal sanitization of medical devices and equipment used in the treatment of hospitalized patients, effective cleaning and disinfection of inanimate contact surfaces is likewise critical for the prevention and control of nosocomial infections within a health care environment. This requirement is especially relevant for hospitalised individuals in the high risk categories i.e. neonates and the elderly, but it also has significance where immunocompromised or patients with heightened susceptibility to opportunistic pathogens require protracted hospitalization.

In developed countries, the control of nosocomial infections is largely driven by political, legal and public awareness, while in developing countries, the prevalence of HIV, malaria, tuberculosis and hepatitis is seen as the primary threat to public health and most HAI's are accorded only a limited significance (Hambraeus, 2006). Correspondingly, it was found that the predominant organisms responsible for nosocomial infections in technically advanced countries were gram positive cocci, while gram negative bacilli were the major cause of HAI's in developing countries (Srivastava and Shetty, 2007).

While the multifactorial origins of nosocomial infections have been extensively assessed, most outbreaks can be traced back to either the lack of effective infection control strategies or their inconsistent implementation (Schabrun and Chipchase, 2006; Srivastava and Shetty, 2007). Coupled to this finding is the assertion that the pursuit of cost containment is contributing to the increasing prevalence of inadequately sanitized surfaces in health care facilities (Griffith *et al*., 2000).

While many disinfecting products are available to the health care market, long term use of many compounds including gluteraldehyde, iodophores and phenols have developed application limitations in terms of carcinogenicity, sensitivity and resistance respectively.

Electrolyzed oxidizing water (EOW), Electrolysed strong acid aqueous solution (ESAAS), Electrochemically activated water (ECA) or superoxidised water (SO), has been reported to be broadly antimicrobicidal with proven bactericidal (including MDR strains), sporicidal, fungicidal, virucidal and cysticical attributes (Venczel *et al*., 1997; Shetty *et al*., 1999; Loshan, 2001; Landa-Solis *et al*., 2005).

There has been a recent increase in the number of reports that describe the benefits of the use of ElectroChemically Activated (ECA) solutions for equipment cleaning and disinfection in the health care arena. In dentistry, the technology has been assessed for the disinfection of dental unit water lines, hand sets and endodontic equipment (Marais and Brözel, 1999; Martin and Gallagher, 2005; Wu *et al*., 2008), and its surface use has also been extended to include invasive disinfection interventions involving root canals (Marais, 2000; Solovyeva and Dummer, 2000). In medicine, the disinfecting ability of the ECA solutions has been reported as being a safe and effective substitute for gluteraldehyde in the cleaning and sanitation of endoscopes and similar frequent re-use equipment in health care facilities (Panicheva, 1999; Selkon *et al*., 1999; Shetty *et al*., 1999; Middleton *et al*., 2000; Thanthsa, 2002; Landa-Solis *et al*., 2005). In addition, it has also been used for more invasive medical treatments and a variety of significant health benefits have been ascribed to its distinctive attributes (Hayashi *et al*., 1997; Nakae and Inaba, 2000; Hanaoka, 2001; Landa-Solis *et al*., 2005).

While there are a number of anecdotal reports on the use of the ECA solutions for environmental decontamination of health care facilities (Devyatov *et al*., 1999; Kruglov and Leonov, 1999; Myazitov and Maximov, 1999; Vorobjeva *et al*., 2004), most reports describe specific ECA interventions under simulated conditions where nosocomial or iatrogenic disease conditions might eventuate (Clark *et al.*,2006).

The oxidant ECA solution has been described as being non-irritating and non-sensitising, free of specific fume extraction or protective clothing requirements, and where disposal and spillage can be managed without special precautions (Marais, 2000; Landa-Solis *et al*., 2005). A further advantage is that the solution can be produced on site, as and when required, thus obviating the traditional problems

associated with logistics, storage, handling and shelf life of packaged chemical products (Middleton *et al*., 2000).

8.3 Objectives of the study

The objective of the study was to establish the antimicrobial efficacy of the Actsol® solution within a high risk medical environment, and to assess the capability of the solution to assist in the control of nosocomial infections due to *Klebsiella spp.* in a neonatal ward. A further objective of the study was to qualify the source and to quantify the prevalence of pathogenic organisms capable of causing nosocomial infection from a variety of surfaces in the NICU and the Postnatal Wards (PNW).

Coupled to this was an investigation to quantify the impact that the introduction of the Actsol® ECA solution as a surface cleaning agent would have on the prevalence of pathogenic surface contaminants, and, finally to compare the relative disinfecting efficacy of the Actsol® solution against the conventional sanitizing procedures and products.

8.4 Materials and Methods

The study was initiated in response to regular disease outbreaks due to *Klebsiella spp*. infections in infants admitted to the NICU. Despite dedicated remedial interventions by the resident infection control authority, *Klebsiella spp*. associated nosocomial infections in the NICU persisted, and an emergency intervention with an initial bulk supply of Actsol® was followed up with the on-site installation of continuously piped supply of Actsol® solution to the NICU sluice room. The ECA device was supplied and maintained by Radical Waters, Kyalami, South Africa.

All equipment including disassembled incubators which were routinely washed in the sluice room were terminally rinsed with Actsol®, and the solution was extensively used for the general surface and floor decontamination in the NICU. In addition, all wash hand basins within the NICU were routinely disinfected with Actsol®.

Due to the medical imperative to introduce Actsol® at the earliest feasible opportunity in order to address the nosocomial outbreaks, the use of the solution in the NICU was initiated four months prior to the approval being granted to conduct the full scale trial in all neonatal and surgical wards. The approved study comprised a thorough microbial prevalence screen of all potentially contaminated in-contact surfaces within the NICU and was undertaken in conjunction with the personnel and facilities of the National Health Laboratory, Gaborone, Botswana.

Initial in-vitro antimicrobial efficacy tests with the undiluted ECA solution indicated that a ten minute exposure period was required in order to achieve maximum disinfection. However the prescriptions of the existing cleaning protocols, the workload of the cleaning staff and the need to limit access to the ICU, required that the exposure period to Actsol® to be shortened to five minutes.

While there is no universal agreement as to the recognized sources of nosocomial pathogens, the ubiquitous focus on hand hygiene has presupposed that horizontal transmission is the primary route by which contamination occurs. In the present study, the use of the anolyte solution was restricted to inanimate objects within the NICU (incubators, working surfaces, floors and drains) and no effort was made to include the use of the oxidant solution for the disinfection of the hands of health care workers, nor of the infant patients themselves.

Designated in-contact surfaces within the NICU were sampled by swabbing to enumerate microbial contamination and to identify the prevalence of pathogenic strains that may result in nosocomial infections in the infant patients. The study was carried out over a period of five months and at least 28 sets of samples were collected on three separate occasions from each surface over the study period. Where comparative assessments of antimicrobial efficacy were possible in the post-natal ward, equivalent sized areas within the ward were separated in terms of type of cleaning chemical to be used, and parallel surface samples were collected for each area. The array of cleaning and disinfecting chemicals used in all wards was comprised of chlorine based products, QAC's and the biguanide based formulation, Chlorhexidine. Investigations indicated that the products were rotated on an ad-hoc basis.

8.4.1 Sample Collection and Analyses

Samples were collected from various surfaces by swabbing a 2 x 5 cm (10 cm^2) surface area with a sterile swab dipped in sterile normal saline (0.9% Sodium Chloride). The swabs were then immersed in 10 ml sterile normal saline and transported to the laboratory. In the laboratory, samples were serially diluted and plated out onto three different media; Plate Count Agar (PCA), MacConkey, and Blood Agar (BA). All media were incubated under aerobic conditions at 37°C for a minimum of 48 hours. On the basis of positive growth on the selective media, suspected pathogenic colonies were isolated and further tested for purposes of identification in accordance with the standard protocols employed by the Microbiology Department of the National Health Laboratory. Microbial enumeration is reported as the Total Viable Count (TVC) and is described by the number of Colony Forming Units per unit sampling area (CFU/cm^2).

The microbial counts for all surfaces sampled were summed and the average microbial count per sampling episode are presented for comparative purposes. No attempt was made to characterize the genotype of the specific strains responsible for the different nosocomial outbreaks and no antibiograms were conducted to establish the antibiotic resistance profile.

8.5 Results

8.5.1 Surface sampling

The results of the initial microbial screen conducted from the various contact surfaces within the NICU and adjacent sluice room, revealed a widespread and consistent degree of contamination by a variety of pathogenic organisms (Table 1). In particular, *Klebsiella spp*. was isolated from most surfaces sampled within the NICU, and displayed a predisposition for moist environments.

This finding accords with the reported partitioning of microbial strains between 'wet' and 'dry' environments in hospital settings, where 'dry-type' sites comprising handles, beds, and curtains were predominantly contaminated by gram positive

organisms, while the 'wet-sites' comprising drains and sinks were contaminated by gram negative bacilli (Gray and Hobbs, 2002; French *et al*., 2004).

Paradoxically, *K. pneumoniae* has been reported to be unstable as an aerosolized fomite at high relative humidity (RH), and displays an enhanced survival and infectious tenacity under conditions of lower RH (Twang *et al*., 2006).

Table 1. Sampling sites and associated microbial pathogens.

Surface	Bacterial strains
Washbasin and Tap lever	*Klebsiella, Pseudomonas, Staphylococcus,*
Floor	*Klebsiella, Staphylococcus, Micrococcus*
Crib	*Staphylococcus, Micrococcus*
Mattress	*Klebsiella, Staphylococcus,*
Shower and Tap lever	*Klebsiella, Pseudomonas, Staphylococcus,*
Toilet Seat	*Klebsiella, Pseudomonas, Staphylococcus, Serratia*

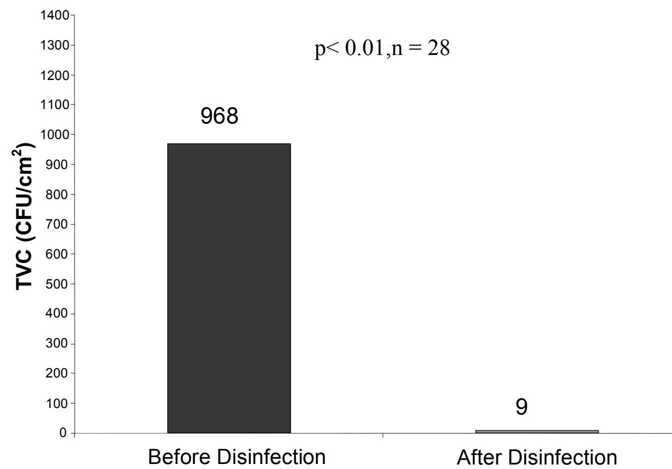
As reported earlier, serious neonatal infections in developing states such as India are predominantly associated with gram negative organisms, and within the NICU, three particular sites were recognized as sources of nosocomial associated pathogens. These comprised of infant incubators and cribs, resuscitation equipment and the various cleaning solutions in use in the facility (Srivastava and Shetty, 2007).

8.5.2 NICU and PNW disinfection

The results of the microbial counts from surfaces in the NICU before and after cleaning with the Actsol® solution clearly demonstrate the antimicrobial efficacy of the oxidant ECA solution when used in a high risk neonatal environment (Fig 1).

The absence of direct comparative data for conventional disinfectant efficacy in the NICU is due to the study being initiated four months subsequent to the first application of Actsol® solution, as was required to address the NICU *Klebsiella spp*. outbreaks referred to earlier.

A direct comparative assessment of the different cleaning efficacies between the standard disinfecting chemicals and Actsol® was only possible in the Post-Natal ward (PNW) and male and female surgical wards. In this aspect of the study, equivalent surfaces were separately disinfected with Actsol® and the standard chemicals.



Legend: TVC – Total Viable Count.

Figure 1. Average NICU surface microbial counts before and after Actsol® disinfection.

The antimicrobial efficacy of Actsol® was further confirmed with the results of the PNW surface swabs, and the substantially persistent microbial load on the surfaces disinfected using standard chemicals clearly indicates a reduced cleaning and disinfection efficacy relative to that of the Actsol® treated surfaces (Fig 2). Disinfection of the NICU with Actsol® reduced the total bacterial count by a magnitude of more than 100 fold (i.e. from an average of 968 to 9 CFU/cm^2). The comparable application of Actsol® in the PNW achieved a 57 fold reduction in the surface microbial count (1488 to 26 CFU/cm^2), while standard chemical disinfection in the PNW achieved an approximately 4 fold reduction (3000 to 733 CFU/cm^2). It is duely recognized that the extensive use of Actsol® in the NICU for four months period prior to the formal study may well have reduced the overall levels of microbial contamination relative to that of the other untreated wards. In addition, the intervention with Actsol®, as well as the extensive sampling and on-site presence may have sensitized the dedicated cleaning staff to the heightened infection control expectations of the trial, and this may further account for the substantive differences in post-cleaning microbial levels between the NICU and the PNW.

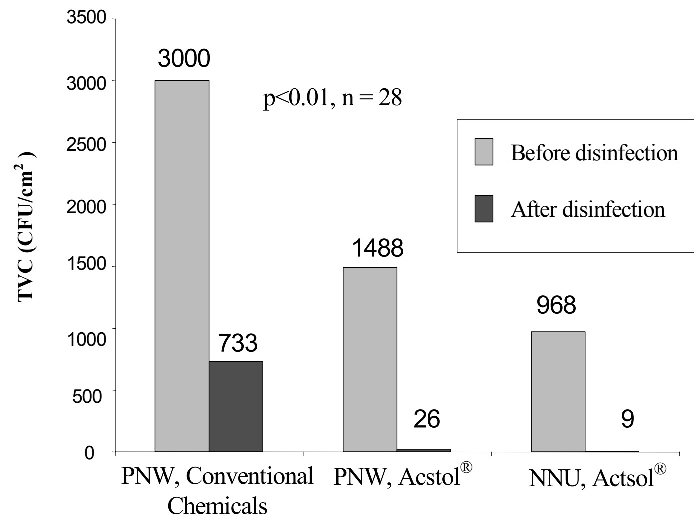


Figure 2. Microbial counts in the NICU and PNW - Comparisons between Actsol® and Standard disinfection.

Anecdotal reports from the nursing staff tended to indicate a higher level of compliance with infection control protocols amongst the cleaners responsible for the NICU relative to those tasked with cleaning the PNW. However, despite these indirect effects, the comparative results for the two disinfectant approaches within the PNW confirmed that the Actsol® solution displayed a substantially increased antimicrobial efficacy as a surface disinfectant relative to that of the standard chemicals employed.

When the results of all three studies are combined, the overall Actsol® antimicrobial efficacy relative to that achieved with the conventional chemical disinfection practices is further substantiated, and the suggested trend of progressive reduction in overall microbial bioload associated with extended Actsol® exposure becomes more tenable.

8.5.3. Extension of the Study to the Surgical Wards

Based on the positive results from the initial phases of NICU and PNW disinfection, the study was extended to the male and female surgical wards to determine whether the observed trends could be duplicated. The same protocol followed within the PNW study phase was adopted, and separate areas were disinfected with the different products. Parallel sampling of equivalent surfaces within each of the two wards was undertaken. Relative to the antimicrobial results obtained in the neonatal wards, the

highly significant differences in antimicrobial efficacy of Actsol® solution relative to that of the standard disinfectants used in the surgical wards suggests that factors other than purely product difference may play a role in justifying the substantive variances (Fig 3).

The equivalent disparity between the results from both the male and female wards confirms the relative superiority of antimicrobial efficacy of Actsol®, but also suggests the role of yet to be determined factors that may account for the difference.

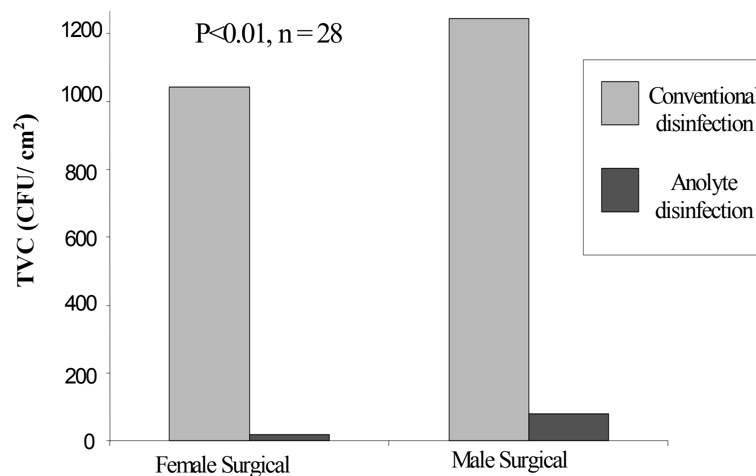


Figure 3. Male and Female Surgical Wards – Comparison between Actsol® and conventional disinfection practices.

8.6 Discussion

The multifactorial aetiology of nosocomial or healthcare associated infections restricts the capacity to accord any single factor as the definitive cause to the event. The dilemma of establishing a casual relationship between impediments in infection control and outbreaks of disease should reflect on the perspective that 'lack of evidence is not evidence of lack' (Griffith, 2006). Given the diverse array of contributing factors, it has been proposed that application of the 'precautionary rule' should be adopted for the resolution of all nosocomial infections (Hambraeus, 2006).

As an extrapolation from the food industry, the precautionary principle states 'where an activity raises the threat to human health or the environment, measures should be

taken, even if the cause-and-effect relationships are not fully scientifically established'(Griffith, 2006).

While neonatal nosocomial infections have been reported to occur from a variety of contaminated sources, it is the infants themselves that are now recognized as being the most frequent reservoir for the horizontal transmission of the pathogens (Casolari *et al*., 2005). This proposal is substantiated by the finding wherein a low level of environmental contamination associated with sporadic enterobacterial disease outbreaks in a neonatal unit, supported the contention that asymptomatic gastrointestinal carriers may be responsible for perpetuating the outbreaks (Gray and Hobbs, 2002; Denton *et al*., 2004).

Other factors that contribute to the risk of nosocomial infections include increased patient turnover and the commercial imperative to have beds filled. Effective manual cleaning and disinfection of complex environments containing beds, furniture and medical equipment is especially difficult under conditions where quick turn-around of facilities are required (French *et al*., 2004). Furthermore, it has been reported that inappropriate nurse to patient ratios and high patient loads relative to available bed space, all serve to heighten the likelihood of HAI's (Hambraeus, 2006). David *et al*., (2006) have detailed a direct correlation between a high bed occupancy rate, elevated patient to nurse ratios and outbreaks of nosocomial infections in NICU's. These studies are substantiated by the report that heavy clinical workloads and extended working shifts, adversely affected hand decontamination practices and resulted in a concomitant increase in patient infection rates (Chudleigh *et al*., 2005).

A review report from 2006 estimated the annual costs associated with nosocomial infections to be of the order of £1 billion in the UK and up to $25 billion in the USA (Schabrun and Chipchase, 2006). In the USA alone, nosocomial infections are responsible for about 1.7 million infections and 99,000 deaths per year (Curtis, 2008). Aside from the direct cost implications, protracted exposure to largely unwarranted chemotherapeutic agents may selectively promote the evolution of tolerant microbial strains that subsequently become established as sources of life threatening diseases (Dancer *et al*., 2006; David *et al*., 2006).

In all too many instances, the liberal use of both systemic and parenteral broad spectrum antimicrobial compounds are relied upon to afford both a preventative as well as therapeutic cover for inadequate sanitization in the health care environment (Srivastava and Shetty, 2007; Borghesi and Stronati, 2008). This overuse of antibiotics has been proposed as one of the main contributors to resistance development (Chapman, 2003), and it has been reported that up to 70% of clinical isolates from hospitals in the USA, are resistant to at least one antibiotic type (Schabrun and Chipchase, 2006).

Apart from antibiotics, the persistence of infectious surface contaminants has also been associated with the widespread use of the broad spectrum disinfectant, chlorhexidine. This disinfectant has previously been reported to promote the development of resistance (Gray and Hobbs, 2002) and Marrie and Costerton, (1981) have reported on the persistent survival of *Ps. aeruginosa* in a 2% chlorhexidine solution for up to 27 months. This phenomenon has also been reported by Denton *et al*. (2004) who describe iatrogenic cross-contamination with a contaminated Quaternary Ammonium Compound (QAC) based product.

Contrary to exploiting the residual properties of disinfectants, it has been proposed that the inadequate removal of biocidal residues after cleaning, only serves to provide a sublethal adaptive platform for the selection of resistant microbial genotypes (Langsrud *et al*., 2003). In a high risk environment, it is fundamental to ensure that the nominated disinfecting compound is applied at dosages which are relevant to the full array of growth profiles which may be present within the contaminating microbial populations. Persistent environmental contamination is likely to be associated with the presence of established biofilms which may support highly resistant and physiologically distinct microbial populations (Lindsay and von Holy, 2006).

Electro-Chemically Activated (ECA) solutions have only recently been introduced for the purposes of disinfection of high risk medical environments. The results of this study confirm the suitability and appropriateness of expanding the use of this technology within this field. While the precise mechanism of action has yet to be described, the microbicidal effect has been ascribed to the high REDOX potential which results in the destruction of the cell barrier without the need or consequences of

the toxic components normally associated with conventional disinfectants (Marais and Brözel, 1999; Middleton *et al*., 2000; Nakae and Inaba, 2000). A study of the antimicrobial efficacy of the ECA solutions confirms that the synergistic activity of the mixed oxidant constituents substantially reduces the minimum microbicidal or inhibitory concentrations that are required relative to that of the equivalent concentrations of direct chemical analogues (Shimuzu and Sugawara, 1996).

In order to interrupt the well established route of infection transfer by contaminated clothing (Hambraeus, 2006), it is imperative that nursing mothers with infants in the NICU should be educated in the principles of barrier nursing and should be provided with gowns to cover their clothing during their stay within the NICU. The 'kangaroo-care' provided by mothers in developing countries is recognized as a cost-effective and widely accepted style of caring for an infant within hospitals. However the role of this care mechanism and its relationship to the incidence of HAI's from a hygiene perspective remains largely untested (Srivastava and Shetty, 2007). The current arrangement for mothers to nurse their infants during hospitalization and their ready access to the NICU, serves to increase the risk of perpetuating the continuous reintroduction of infectious agents into the NICU.

Discussions with hospital personnel prior to this study confirmed the perception that inadequately sanitized hands remains the single biggest factor for the spread of infections within the hospital. It has been established that there is a strong correlation between homologous hand contamination and the infectious organisms involved in nosocomial infections (Denton *et al.,* 2004). Chudleigh *et al.* (2005) have noted that optimal hand decontamination is considered the most important means of preventing healthcare associated infections, and that the frequency of hand decontamination was substantially less important than the basic hand washing technique in terms of overall disinfection efficiency (Lewis *et al*., 2008). As an adjunct to the surface disinfection study, the microbial contamination of the hands of both mothers and staff associated with the NICU was assessed. Whilst only a superficial screen, the results showed that more than 60% of persons sampled carried pathogenic organisms on their hands after routine washing.

In resource restricted settings, advice on the requirements for optimal hand washing as well as choice of the appropriate antimicrobial agent are substantially more important than the expensive epidemiological assessments that inevitably follow disease outbreaks (Srivastava and Shetty, 2007). Kruglov and Leonov (1999) have reported on the beneficial effects of using the ECA solutions for the disinfection of health care workers hands, and while equivalent in antimicrobial efficacy to 70% alcohol based scrubs, anecdotal evidence suggests that fewer incidents of irritation, skin cracking and dryness with the ECA solutions may translate into enhanced compliance with hand hygiene requirements.

8.7 Conclusions

The near term and sustained reduction of NICU surface microbial counts by the Actsol® solution would suggest that direct contact with contaminated inanimate surfaces was primarily responsible for the original *Klebsiella spp.* infection and that the subsequent cross-contamination of other infants was due to a secondary horizontal hand and/or equipment based transmission. While the infectious focus of the *Klebsiella spp.* appears to have been restricted to nurse's hands and the drains in the sluice room adjacent to the NICU, it is also feasible that the traditional 'kangaroo-care' style of maternal nursing practiced in the NICU may also have perpetuated the inadvertent pathogen transmission by the mothers during their frequent visits into the NICU.

In a resource poor environment it is recognized that a well structured and workable infection control strategy is essential, but that it must have secured universal 'buy-in' in order for it to be sustainable. In developing countries, the most common route for the spread of neonatal nosocomial pathogens is person-to-person transmission within the unit, and the most common iatrogenic factor contributing to neonatal HAI's remains the hands of healthcare workers. Resources, experience, understanding of procedures and commitment toward infection control compliance are recognized as being the fundamental criteria for the control of nosocomial infections in Neonatal Intensive Care units.

The use of Actsol® as a safe and effective surface disinfectant in high risk intensive care facilities was confirmed. On the basis of the positive comparative antimicrobial results, it is proposed that it may readily be used as an alternative to standard disinfecting practices.

8.8 Acknowledgements

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Chapter 9

Antimicrobial efficacy of Actsol®[1], an Electro-Chemically Activated (ECA) oxidant solution against multi-drug resistant bacteria.

9.1 Abstract

The antimicrobial efficacy of Actsol®, an Electrochemically Activated (ECA) oxidant disinfectant produced by Radical Waters, South Africa, was evaluated against a range of hospital Multi-Drug Resistant (MDR) bacterial isolates using a standard *in-vitro* suspension method. The product was tested both with and without the addition of 1% horse serum to evaluate the anti-oxidant effects of bio-soiling. Clinical isolates of MDR bacteria were obtained from the National Health Laboratory Services (N.H.L.S) Microbiology Laboratory at the Chris Hani Baragwanath Hospital, Gauteng, South Africa. These isolates comprised both Gram-positive and Gram-negative bacteria with variable antibiotic resistance profiles, and all strains were recognised as being common nosocomial pathogens within the hospital environment. This study confirmed the excellent broad spectrum bactericidal properties of Actsol® even in the presence of bio-soiling. A marginal reduction in biocidal efficacy was observed when the diluted Actsol® solutions were tested in conjunction with 1% horse serum. There did not appear to be any relationship between the antibiotic resistance profile of the various strains of bacteria and susceptibility to the Actsol® solutions.

9.2 Introduction

The HIV/AIDS pandemic in Africa is placing an ever-increasing burden on the continent's health services. This can be characterised as an increase in the number of patients attending and sojourning in already overcrowded hospitals, a prolonged treatment time, multiple concurrent infections particularly with micro-organisms previously considered as having a low pathogenicity and an increased susceptibility of patients to nosocomial infections where a lower infective dose is required to cause patent clinical disease.

1- Actsol® is the registered trademark of Radical Waters (Pty) Ltd.

In addition to these exaggerated epidemiological pressures, is the concern that while disinfectants remain a cornerstone of any infection control policy designed to limit the spread of pathogenic microorganisms, recent developments have suggested the evolution of co-resistance between disinfectants and antibiotics, where the injudicious use of disinfectants may progressively increase the prevalence of pathogenic MDR strains (Suller and Russell, 1999).

It has been reported that there is a general increase in the number of multi-drug resistant organisms being isolated world-wide (National Nosocomial Infections Surveillance System, 1999). This problem is exacerbated where patients require protracted hospitalisation or where multiple concurrent disease conditions and diminished immunocompetence result in a suboptimal clinical response to chemotherapeutic interventions. This progressive accumulation of MDR strains also significantly increases the risks to attendant health care workers.

A generalised association between antibiotic resistance and reduced susceptibility to disinfectants has been recently reported (Wisplinghoff *et al*., 2007). While reports on chlorhexidine resistance has been shown ever increasingly to be associated with both Methicillin Resistant *Staphylococcus aureus* (MRSA) and Vancomycin Resistant *Enterococcus* (VRE), the authors concluded that resistance to concurrently used disinfectants was not a risk factor in the spread of nosocomial infections and that all the commonly used disinfectants assessed were able to inhibit growth of MDR strains at the recommended concentrations and exposure times.

While resistance to disinfectants was originally ascribed to intrinsic chromosomally encoded responses, extra-chromosomal, plasmid encoded resistance to non-antibiotic agents is now a widely reported phenomenon and specific studies have linked the presence of resistance to chlorhexidine and Quaternary Ammonium Compounds (QACs) to the repeatable presence of plasmids (Russell, 1997).

Resistance to disinfectants presents mainly as an increase in the Minimum Inhibitory Concentration (MIC) required for optimal inactivation, and reduced biocidal sensitivity of Epidemic MRSA or EMRSA strains has been reported to display a 30-

50 fold increase in the MIC of sodium hypochlorite relative to that of Methicillin Sensitive *Staphylococcus aureus* (MSSA) strains (Mycock, 1985).

A diverse array of bacterial tolerances to disinfecting agents has been reported. Aldehyde and biguanide based formulations and the QACs have been shown to display limited anti-mycobacterial activity while the Anilides have been reported to display limited activity against Gram negative bacteria and fungi. The biguanides (e.g. Chlorhexidine) have been reported to lack sporicidal activity, and the bisphenols and halophenols have been shown to have limited activity against the Pseudomonads, Enterobacteriaceae, non-fermenters and moulds (Suller and Russell, 1999; Putman *et al*., 2000; Loughlin *et al*., 2002; Chapman, 2003). Similarly, peroxygen compounds (e.g. Virkon®) have been shown to display limited fungicidal and sporicidal activity, and only exhibit mycobactericidal and virucidal activity in the absence of organic soiling and then only at certain minimum concentrations (Garćia-de-Lomas *et al*., 2008). While there are a limited number of reports suggesting resistance to oxidising agents, a recent study has described an equivalent adaptive reponse to low dose chemically generated hypochlorous acid to that induced by hydrogen peroxide exposure (Mokgatla *et al*., 2002).

However, contrary to these extensive survey data, Wisplinghoff *et al*. (2007) was unable to describe any definitive correlation between antibiotic resistance and decreased disinfectant susceptibility in *Acinetobacter baumannii* at the recommended usage recommendations - this despite widespread suggestions of a directly causal relationship in other gram positive and negative bacterial strains.

Previous reports on the antimicrobial efficacy of Actsol® (Marais and Brözel, 1999; Marais, 2000; Cloete, 2002) or similar ECA based solutions (Selkon *et al*., 1999; Shetty *et al*., 1999; Fenner, 2005) have shown that the product is substantially broad spectrum in activity, as well as being rapidly bactericidal with cell lysis being evidenced within 25 seconds of exposure (Zinkevich *et al*., 2000). Actsol® has been shown to remain stable for up to 6 months under optimal packaging and storage conditions (Radical Waters, Unpublished data). Previous studies conducted in the UK (Selkon *et al*., 1999, Shetty *et al*., 1999) show that it is non- toxic to cells in tissue culture as well as being non-mutagenic (Bakhir *et al*., 2003; Panichev, 2006). *In–vitro*

tests performed by Biocon Research (Pty) Ltd., a South Afrcian laboratory accredited under the guidelines of the FDA, have confirmed that Actsol® is not cytotoxic and non-sensitising to animal cells, in addition to being a highly effective, broad spectrum antimicrobial agent (Marais, 2002).

In an effort to describe a possible causal relationship between MDR and the purported resistance to disinfectants, as well as to reaffirm the different antimicrobial properties between an electrochemically activated oxidant solution and a commercially available hypochlorite preparation, the comparative antibacterial efficacy study was conducted using a variety of confirmed bacterial MDR hospital isolates. To this end, the largely inconclusive reports which to date have suggested a causal relationship between multi-drug resistance and concurrently used disinfectant compounds, has warranted further elucidation specifically with regard to the evaluation of the antimicrobial efficacy of the ECA technology. Additionally, the oxidant neutralisation effect which is conventionally associated with the presence of organic soiling, and the consequential impact on the potential development of resistance to the ECA solutions was also included in the assessment.

9.3 Materials and Methods

9.3.1 Description of Actsol®

Actsol® is the oxidant component of Electro-Chemically Activated (ECA) water generated from a dilute saline solution that has been passed through a powerful electrical field. It is a highly positively charged, mixed oxidant solution and has been reported to be comprised of predominantly hypochlorous acid, chlorine dioxide, hypochlorite, ozone, hydrogen peroxide, and a variety of metastable radicals including hydroxyl, superoxide and singlet oxygen (Prilutsky and Bakhir, 1997; Bakhir, 1999). The prescribed device configuration and operational parameters required to produce the specific ECA solutions has been extensively described in previous chapters.

9.3.2 Source of bacterial strains

Strains of multidrug-resistant bacteria were obtained from the Microbiology Laboratory of the National Health Laboratory Services (N.H.L.S.) at the Chris Hani Baragwanath Hospital (CHBH) in Gauteng, South Africa. Owing to the non-availability of a vancomycin-resistant *Enterococcus* (VRE) strain from the Hospital wards during the course of this study, an American Type Culture Collection (ATCC) strain was secured from an alternative source. The *Pseudomonas aeruginosa* strain which was not highly resistant, but which had an antibiogram profile representative of strains frequently isolated in the wards was also included.

The bacterial strains evaluated in this study comprised of *Acinetobacter baumannii* (5 strains), *Escherichia coli* (4 strains), *Ps. aeruginosa* (7 strains), *Enterobacter* sp. (1 strain), *Salmonella* sp. (1 strain), *S. isangi* (1 serotype), *Klebsiella* spp. (5 strains), *Staphylococcus aureus* (6 strains), *Enterococcus faecium* (2 strains) and *E. faecalis* (1 strain) (Appendix 1 and 2). The array of bacterial strains were recognised to be representative of the organisms commonly encountered during laboratory isolation, and most isolates were prepared from specimens received from patients from the paediatric and respiratory Intensive Care wards.

The bacterial strains were maintained in semisolid agar (N.H.L.S., South Africa) after laboratory isolation and were sub-cultured no more than twice prior to this study in order to maintain the integrity of the antibiotic resistance profile.

As detailed in Table 3, most gram negative bacilli displayed Extended Spectrum β Lactamase (ESBL) activity as well as concurrent High-Level Aminoglycoside resistance. ESBL Salmonellae isolated from the hospital were shown to belong predominantly to the *S. isangi* serotype. MDR MRSA strains (Table 4) were included due to their capacity to produce both catalase and superoxide dismutase, and it was speculated that these mechanisms may have played a protective role during exposure to the hydrogen peroxide and hydroxyl radicals present in the Actsol® solutions.

Ps. aeruginosa is widely reported to be associated with resistance to disinfectants. This has been documented both within hospital isolates as well as those strains

commonly found in the water treatment industry (Brözel, 1992). This study comprised ward isolates which reflected the full array of antibiotic resistance profiles routinely encountered within the hospital (Table 3). Oxidant disinfectants such as hypochlorite are known to display diminished antimicrobial performance in the presence of organic soiling. The addition of 1 % horse serum (N.H.L.S., Virology Department, Rietfontein, South Africa) to the assessment protocol, was included in an attempt to quantify the degree to which the antimicrobial efficacy of the hypochlorite preparation as well as the Actsol® solution would be compromised by the presence of organic matter.

9.3.3 Test conditions, Exposure time and Neutralisation

Actsol® solution was supplied by Radical Waters (Gauteng, South Africa) and was generated from a 0.25% NaCl stock solution, using a previously described FEM based reactor device. The device was preset with a power rating of 12v and 5A and a flow rate of 350ml/min per FEM reactor unit. The range of physicochemical parameters of the different Actsol® solution used in the assessment are characterised in table 1.

Table 1. Physicochemical parameters of the different solutions used in the antimicrobial efficacy assessment.

Parameter	ORP (mV)	pH	EC (mS/cm)	FAC (mg/L)*
Hard Water	235	9.6	0.71	0
Horse Serum	330	7.2	11.4	0
Jik®	550	10.8	1.09	240**
Jik® + Horse serum	515	1.07	1.17	180
Actsol® solutions				
Undiluted	910	6.7	4.92	170
Undiluted + Horse serum	956	6.7	5.02	170
1:10	460	9.7	1.13	10
1:10 + Horse Serum	462	9.0	1.24	10
1:100	263	9.8	0.74	1
1:100 + Horse serum	240	9.6	0.91	1

Legend: ORP- Oxidation Reduction Potential (milliVolts), EC – Electrical Conductivity (milliSiemens per cm), * - FAC: Estimated Free Available Chlorine (milligram per litre), ** - as per manufacturer recommended dilution.

The antimicrobial efficacy of the Actsol® solution was assessed using both the undiluted solution, as well as 1:10 and 1:100 dilutions using standard hard water (0.15g Calcium Chloride, 0.15g Magnesium Chloride in 1000 ml distilled, deionised water) obtained from the N.H.L.S. as the diluent.

Commercially available sodium hypochlorite (Jik®: 3.5% m/v) was used as the alternative chlorine-based comparison, and was formulated to the recommended working strength (i.e. 240mg/L Free Available Chlorine [FAC]) as advised by the manufacturers (Reckitt-Benckiser, SA). The FAC of all solutions was determined using Merckoquant® Chlor-test kits for the 0-20 and 0-500mg/L concentration ranges (Merck, SA). The sodium hypochlorite solution and the Actsol® were used both undiluted, as well as diluted to a 1:10 and 1:100 strength using standard hard water. A control to determine viability count was included, and the Actsol® solution was replaced with a distilled, deionised hard water control and all tests were performed in duplicate.

The procedure for the preparation of the innocula was in accordance with the standard procedures detailed in the guidelines of the South African Bureau of Standards (SABS) (1999). After extraction from the semi-solid agar, all test cultures were grown on standard Mueller-Hinton agar plates (Difco, Detroit, Michigan, USA) at 37°C for 18 hours. Colonies were collected and suspended in distilled water using a sterile metal spreader to give an approximate concentration of 1.0 x 10^8cfu/ml by comparing the opacity with a McFarland's 0.5 $BaCl_2$ opacity standard (Koneman *et al*., 1997). A 0.5 ml aliquot of the suspension of each organism was added to 4.5 ml of either the Actsol® or sodium hypochlorite test solution in a sterile screw top plastic test tube, therein yielding a final bacterial load of 1 x 10^7 cfu /ml.

All test organisms were exposed to the array of biocide solutions for 5 minutes. Where the 1.0% horse serum was included, the serum was first added to the Actsol® solutions, and then thoroughly agitated prior to the addition of the challenge dose of the different bacterial strains. This step was followed in light of the previously reported rapid bactericidal action of the Actsol® solution which may have biased the interpretation of any soil neutralisation effect had it been added after the bacterial challenge (Zinkevich *et al.*, 2000).

Upon termination of the exposure period, the tubes were rapidly inverted 3 times to ensure homogenous distribution of all test organisms. Thereafter, 0.5 ml of the suspension in each of the test solution permutations was removed and added to 4.5 ml of a 1% (m/v) sodium thiosulphate solution, and the tube was inverted several times for a 1 minute period to ensure neutralisation of any residual disinfecting agent. This served to effect a further 10 fold dilution to the challenge aliquot. After the exposure to sodium thiosulphate, a 1 ml aliquot was removed and spread on the surface of Mueller-Hinton agar plates with a sterile metal spreader. All tests were carried out in triplicate and the plates were then allowed to dry for 1 hour before transfer to the incubator. All plates were incubated at 37°C for 48 hours. After incubation, all plates with discrete colonies were enumerated and the mean counts of the colony forming units (CFU) were recorded.

9.4 Results

As detailed in Table 2, the undiluted as well as the 1:10 Actsol® dilution inactivated all organisms within the 5 minute exposure period in the absence of horse serum. The 1:100 Actsol® dilution inactivated all organisms with the exception of the mucoid *Ps. aeruginosa* strains where a 6 log_{10} reduction was achieved. A direct comparison with the results of the exposure of the bacteria to the commercial sodium hypochlorite solution without the addition of horse serum revealed a substantially equivalent antimicrobial efficacy.

While the addition of horse serum to the diluted Actsol® solutions did reduce the overall inactivation efficiency, the undiluted Actsol® solution mixed with 1% horse serum still achieved a complete inactivation rate despite the presence of the simulated biosoiling. The highly mucoid strains of *Ps. aeruginosa* were shown to display the greatest measure of tolerance to the diluted and soiled Actsol® solutions. Both the undiluted Actsol® and the working strength Jik achieved a 7 log_{10} kill against these mucoid strains.

Organisms producing catalase (i.e. *S. aureus*) did not display any diminished inactivation when compared against the catalase-negative *Enterococci*, despite the presence of reactive hydroperoxy radicals in the Actsol® solution. Gram negative

bacilli appeared to display greater overall sensitivity to the Actsol® solutions relative to the gram positive coccal strains.

Table 2. Average reduction in microbial log count after exposure to various Actsol® dilutions and sodium hypochlorite both with and without horse serum.

Disinfectant and dilution series	Actsol®			Actsol® + HS			Jik®	Jik®+HS
	100%	10%	1%	100%	10%	1%	As recommended	
Gram negative bacterial strains								
A.baumannii	100	100	100	100	99.99	99.0	100	100
Ps.aeruginosa	100	100	100	100	99.99	99.9	100	100
E.coli	100	100	100	100	100	99.9	100	100
Enterobacter spp	100	100	100	100	100	99.9	100	100
Klebsiella spp.	100	100	100	100	99.999	99.9	100	100
Salmonella spp	100	100	100	100	99.9	99.9	100	100
Gram positive bacterial strains								
E. faecalis	100	100	100	100	99.9	99.0	100	100
E. faecium	100	100	100	100	99.9	97.0	100	100
S. aureus	100	100	100	100	99.9	99.0	100	100

Legend: HS – 1% Horse serum,

9.5 Discussion

Resistance refers to the condition where a bacterial strain is not killed or inhibited by a concentration of biocidal agent to which the strains of that same organism had previously been shown to be susceptible (Russell, 2001).

The bacterial cell is comprised of three distinct components - the genome, the cytoplasm and the outer barrier structure (Brözel, 1992). Of these three, it is widely recognised that the integrity of the barrier is fundamental to the survival of the cell, and that its specific composition and structure confers distinctive abilities to counter the adverse effects of diverse biocidal challenges. Additionally the stage of growth, participation in a biofilm consortium, changes in nutrient availability as well as temperature have all been shown to influence the relative proportions of the different constituents of the cell wall or membrane. This will in turn, directly influence the

barrier properties of the same, and consequently, the ability of the given bacterium to withstand the potentially adverse impact of physical and chemical agents.

Fundamentally, the condition of resistance arises due to either an insufficient quantity of the biocidal agent being presented at the target site, or the increased capacity of the bacteria to externalise or degrade noxious agents and to repair the damage that they may inflict. Resistant states have been reported to arise from a reduced uptake of the antibacterial agent, extracellular neutralisation of the biocide by the presence of the superficial alginate, an increase in cytoplasmic degradatory enzymes, a change in the biocide target site to a non-susceptible state and the induction of efflux mechanisms that cause the expulsion of the noxious chemical agents (Brözel, 1992; Russell, 1997; Cloete, 2003).

Given that neutral ECA anolyte is predominantly comprised of hypochlorous acid, the report by Mokgatla *et al*., (2002) wherein a *Salmonella* isolate was shown to respond to a low dose hypochlorous acid exposure within 10 minutes by inducing increased catalase production is the first indication of potential resistance to ECA solutions. Hypochlorous acid is reported to be a highly destructive and non-selective oxidant that readily reacts with a variety of subcellular compounds to disrupt metabolic processes (Dukan and Touati, 1996). That the low dose hypochlorous acid had been reported to cause severe and progressive DNA disruption within 3 minutes of exposure, would support the suggestion that the autocidal generation of secondary ROS was responsible for the consequential disruption arising from the initial insult. Additionally, it was shown that the increased levels of catalase activity in the hypochlorous acid tolerant strain resulted in a two fold increase in the degradation of hydrogen peroxide relative to that of the hypochlorous acid sensitive strain. This response would suggest that there may have been a non-specific induction of an intrinsic adaptive mechanism which was geared primarily to a cellular reponse normally mediated by hydrogen peroxide. It must be emphasised that the hypochlorous acid used in this study was not generated by an electrolytic process and hence did not have the synergistic biocidal benefits of the elevated REDOX potential previously described (Prilutsky and Bakhir, 1997, Bakhir, 1999, Marais, 1999; Zinkevich, 2000). In the absence of an elevated REDOX exposure, it is thus proposed that the resistant isolate was able to protect itself against exposure by decreasing the

levels of reactive species which could be expected to react with hypochlorous acid to generate toxic reactive oxygen radicals, and that the mechanism of oxidative tolerance was due to a combination of physiological adaptations which collectively led to an enhanced degree of tolerance (Mokgatla *et al*., 2002).

The role of extracellular barrier mechanisms to neutralise the non-selective and highly reactive oxidant species in the Actsol® solutions is supported by the enhanced sensitivity displayed by the non-mucoid strain of *P. aeruginosa* relative to that of the two mucoid isolates when tested at a 1:10 dilution in the presence of horse serum. This differential biocidal effect was shown to be independent of the antibiotic resistance profile of the different strains, as all isolates displayed a substantially equivalent MDR profile. It is thus proposed that the oxidant chloroxy and hydroperoxy radicals of the Actsol® solutions may have reacted with the alginate of the mucoid *Ps. aeruginosa* strains and that the latter acted as a sacrificial antioxidant to the mixed oxidants in the Actsol® solution, thereby leaving the core underlying cell structures intact.

While it is acknowledged that certain correlates may be drawn between MIC derived disinfectant resistance profiles and the presence of specific genes that have been reported to encode for the efflux of the same categories of biocidal agents, the limited conclusions drawn may not necessarily reflect the broader and more holistic dictates that govern the survival of a biocide challenged microorganism.

Commentary on the validity of the use of manufacturer MIC's as definitive descriptors of states of tolerance or resistance have been questioned, and it has been suggested that laboratory based resistance determinations may, at best, be tenuous. Relative to the selection pressure exerted by intensive antibiotic use and the consequential selection of adaptive geno- and phenotypes, the claims of purported resistance to disinfectants may be viewed as trivial, and it has been proposed that the rate of inactivation rather than the degree of inhibition is a substantially more relevant indicator of clinical bacterial susceptibility to disinfectants (Suller and Russell, 1999). Despite the vast number of reports that suggest otherwise, the efflux of a broad range of structurally unrelated toxic compounds may also be viewed as a consequence of a

normal primary physiological function and thus a fortuitous side effect of the transport of a common physiological or metabolic substrate (Putman *et al*., 2000).

Aside from its proven antibacterial efficacy, direct exposure to Actsol® solutions has shown that it is non-cytotoxic to skin, mucous membranes and the conjunctiva, and that it does not precipitate skin hypersensitivity reactions in guinea pigs, rats or rabbits (Marais, 2002; Bakhir *et al*., 2003). It has been proposed that the substantially safer exposure profile is due to the action of low levels of hypochlorous acid (HOCl) which closely mimics the mechanisms of the mammalian leucocyte based antimicrobial system (Cunningham and Ahern, 1995).

In the undiluted state, reasonable levels of soiling (equivalent to 1% horse serum) did not impair the antimicrobial efficiency of the electrochemically activated Actsol® solution. Additionally, the antimicrobial efficacy of the undiluted Actsol® solution was shown to be directly equivalent to the commercial hypochlorite solution, albeit that the FAC concentration of the two solutions was 170 and 240mg/L respectively.

The choice of standard hard water as a diluent for the oxidant Actsol® solution resulted a substantial reduction in the ORP of the diluted solutions. This is attributed to the elevation in pH and the conversion of the available chlorine into the less microbicidal hypochlorite moiety which may have limited the antimicrobial efficacy of the diluted Actsol® solutions. It is proposed that the reduction in the REDOX potential without any significant change in FAC concentration was primarily responsible for the substantial reduction in antimicrobial efficacy.

9.6 Conclusions

This preliminary study indicates that Actsol® could be a useful disinfecting agent for reducing the incidence of nosocomial outbreaks in health care facilities, and hence, is an effective tool to manage the development of multi-drug resistant strains commonly associated with these persistent nosocomial infections. A rapid bactericidal effect against both gram-positive and negative MRD bacterial strains was achieved even in the presence of simulated biosoiling

Despite a substantially lower active reagent concentration (FAC), the neutral anolyte was able to achieve an equivalent degree of bacterial inactivation to that of the commercial hypochlorite over the same exposure periods. Both the electrolysed and chemical oxidant solutions were able to inactivate all strains of bacteria irrespective of their antibiotic resistance profiles and the results would suggest that there is no cross or co-resistance between the encoded antibiotic resistance and the oxidant species present in the two test solutions.

These results confirm that Actsol® is an effective alternative biocidal agent against most bacteria including MDR strains and that it retains its antibacterial efficacy in the presence of low levels of organic material.

9.7 Acknowledgements

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9.10 Appendices

Appendix 1. Antibiotic resistance profile of gram negative bacterial isolates evaluated in the study.

Gram Negative Organisms	Imperilling	Co-amoxyclav.	Piperacillin	Pip-taz	Cefazolin	Cefuroxime	Ceftriaxone	Ceftazidime	Cefepime	Imipenem	Meropenem	Gentamicin	Amikacin	Tobramycin	Ciprofloxacin	Cotrimoxazole	Chloramphinicol
P.aeruginosa (M)			s	s				s	s	r			s		s		
P.aeruginosa (M)			r	r				r	r	r	r	r	r		r	r	r
P.aeruginosa			s	s				s	s	s	s	r	s		s	r	r
P.aeruginosa			r	r				s	s	s	s	r	r		r	r	
P.aeruginosa			r	r				r	r	r	r	r	r		r	r	s
P.aeruginosa			s	s				r	s	r	r	r	r		r	r	r
P.aeruginosa			r	r				r	r	r	r	r	r		r	r	r
A.baumannii	r	r	r	r	r	r	r	r	r	r	r	r	r	s	r	r	
A.baumannii	r	r	r	r	r	r	r	r	r	s	s	r	r	r	r	r	
A.baumannii	r	r	r	r	r	r	r	r	p	s	s	r	r	r	r	r	
A.baumannii	r	r	r	r	r	r	r	r	r	r	r	r	r	s	r	r	
A.baumannii	r	r	r	r	r	r	r	r	r	r	r	r	r	p	r	r	
A.baumannii			r	r				r	r	r	r		r	s	r		
A.baumannii			r	r				r	r	s	s		r	r	r		
S. isangi	r	r		s			r			s					s	r	
Salmonella sp.	r	r	r	r	r	r	r	r	r	s	s	r	s	r	s	r	
Enterobacter sp.	r	r	r	r	r	r	r	r	r	s	s	r	r	r	s	s	
E.coli	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
E.coli	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
Klebsiella sp.	r	r	r	r	r	r	r	r	r	s	s	r	s	r	s	s	
Klebsiella sp.	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
Klebsiella sp.	r	r	r	p	r	r	r	r	r	s	s	p	r	r	s	r	
Klebsiella sp.	r	r	r	s	r	r	r	r	r	s	s	r	s	r	s	r	
Klebsiella sp.	r	r	r	r	r	r	r	r	r	s	s	r	s	r	s	r	

Legend: r – Resistant; s – Sensitive; p – partially resistant ; (M) – Mucoid. (Resistance type: *A. baumannii* – ESBL and AME; *E coli* –ESBL; *S. isangi* – ESBL; *Enterobacter* sp. – ESBL; *Klebsiella* spp – ESBL)

Appendix 2. Antibiotic resistance profile for gram positive bacterial isolates evaluated in the study.

Gram positive Organisms	Pen/Ampicillin	Oxacillin	Erythromycin	Clindamycin	Tetracycline	Co-trimoxazole	Rifampicin	Vancomycin	Fucidin	Ciprofloxacin	Chloramphenicol	Gentamycin high level conc.
MRSA	r	r	r	s		r	s	s	s	r		
MRSA	r	r	s	s		r	r	s	s	s		
MRSA	r	r	r	s		r	r	s	s	s		
MRSA	r	r	r	r		r	r	s	s	r		
E. faecium	r	r	r	r	s	r	r	s	s	r	r	r

Legend: r – Resistant; s – Sensitive.