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^{o}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^{o}C), mesophiles (25-45^{o}C) and psychrophiles (<20^{o}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^oC, (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.

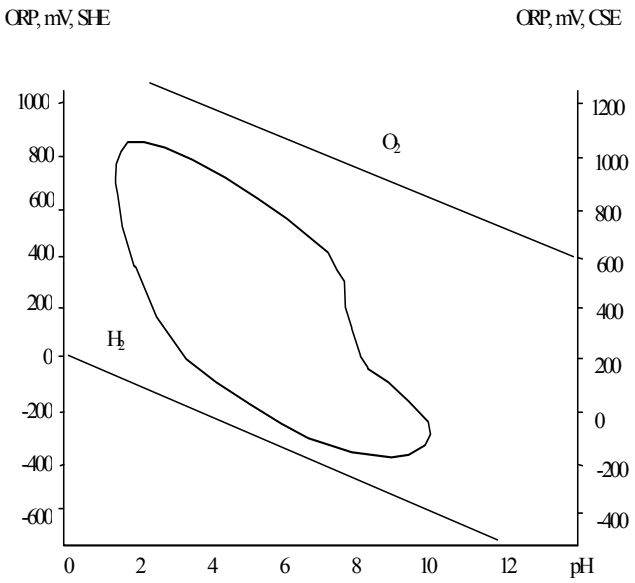


Figure 1. Range of pH and ORP values capable of sustaining microbial growth in artificial media (Prilutsky and Bakhir, 1997).

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.

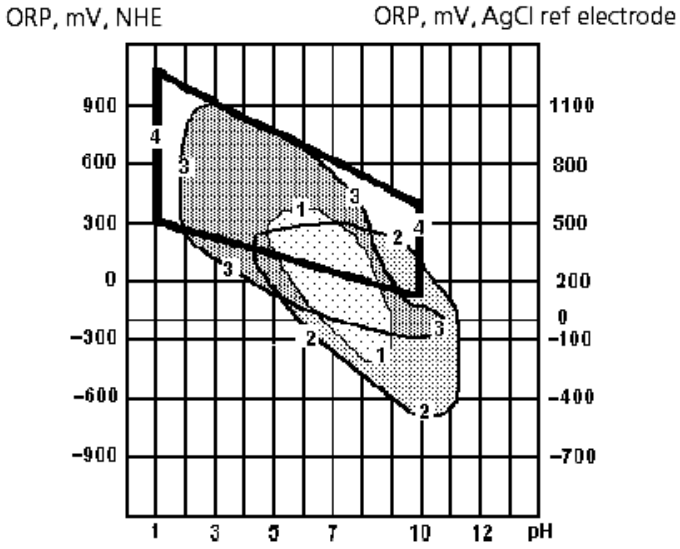
Table 2. Correlation between pH and ORP and the measure of the range of ORP values tolerated for growth of different microbial types (Prilutsky and Bakhir, 1997).

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).

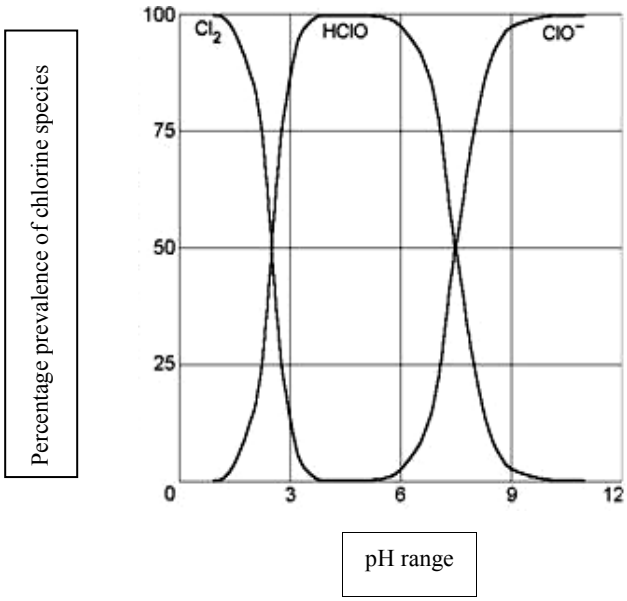

Figure 1. Prevalence of chlorine and oxy-chlorine species in aqueous solution as a function of pH. (Bakhir *et al*., 2003)

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.

3.6 References

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