The effect of anolyte and a combination of anolyte and catholyte on biofilms

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INTRODUCTION

Adhesion to surfaces is a common and well known behaviour of microorganisms in oligotrophic habitats (Zobell, 1943). This adhesion and subsequent metabolism lead to the formation of biofilms (39). Bacterial biofilms promote increased biomass deposition (54), resulting in fluid flow resistance, loss of heat exchange and microbial induced corrosion in industrial water cooling systems (13).

Industries control unwanted biofilms, with varying degrees of success, by using biocides (13). The use of biocides, especially chlorine, in water reticulation and heat exchange systems is effective only if the biofilm is removed manually. Chlorination of a mature biofilm is usually unsuccessful because the biocide only reacts with the outer portion of the biofilm, leaving a healthy and substantial bacterial community on the surface that rapidly regrows (10). Bacteria within biofilms develop increasing resistance to the biocide on repeated dosing (13). Brözel and Cloete (10, 11, 12) found that biocides also induced cross-resistance to other biocides.

Microbial biofilms are problematic in a range of industrial environments where large areas of submerged surfaces are exposed to relatively high nutrient fluxes, providing niches for the formation of copious surface-associated growth (13, 18). Bacterial colonisation of surfaces in an aqueous environment is a basic strategem for survival in nature as nutrients are more available at the solid - liquid interface (27, 32). The resulting aggregates form microcolonies which develop into biofilms (39). Biofilms increase fluid frictional resistance (39) and decrease the rate of heat energy transfer, collectively termed biofouling. These biofilms also promote corrosion of ferrous and other metals by the concerted metabolic activity of a number of biofilm-associated bacterial types (38), a process collectively termed microbially influenced corrosion (MIC). MIC encompasses a number of specific mechanisms relating either directly or indirectly to the metabolic activity of a variety of microorganisms, notably the action of sulphidogenic bacteria (19, 33). As the costs attributable to MIC and biofouling are high, effective control of bacterial numbers in industrial aqueous environments is essential.

A range of bactericidal substances, commonly termed biocides or microbicides, are available, all of which are claimed by their producers to kill bacteria occurring in aqueous systems quantitatively. Biocides target a range of cellular loci, from the cytoplasmic membrane to respiratory functions, enzymes and the genetic material (47). However, different bacteria react differently to bactericides, either due to inherent differences such as unique cell envelope composition and non-susceptible proteins (9), or to the development of resistance, either by adaptation or by genetic exchange (11). Bactericides should therefore be evaluated against the organisms which they are chosen to control, i.e. the dominant ones in the system to be treated. The composition of microbial populations in systems varies with the type of water used, and changes considerably following treatment with various biocides by selection for resistant strains (10). Bacteria growing as biofilms are also significantly more resistant to most antimicrobial agents known currently, so that methods for their control pose an ongoing challenge (14, 18)

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

Five approaches are currently available and may be used in combination:
(i)   bacteria are chemically killed by application of biocides;
(ii)   cells are dislodged from surfaces by dispersants;
(iii)   the biofilm structure is weakened by enzymes or chelants of divalent cations;
(iv)   biofilms are removed physically by a variety of processes, and
(v)   biocide efficacy is enhanced by applying an alternating current or ultrasonic sound across the biofilm.

A novel way of the electro chemical activation of water was recently introduced in South Africa. During Electro Chemical Activation (ECA) of water, a dilute saline solution is "activated" by passing through a cylindrical electrolytic cell in which the anodic and cathodic chambers are separated by a permeable membrane. During the process of electrochemical activation three broad classes of product are produced:

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

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

Two separate streams of activated water are produced: Anolyte with a pH range of 2-9 and an oxidation- reduction potential (ORP) of +400 mV to +1200 mV. Anolyte is an oxidizing agent due to a mixture of Free Radicals and has an antimicrobial effect. Catholyte with pH of 12 to 13 and an ORP of about –900mV. It has reducing and surfactant properties and is an antioxidant.

The aim of this study was to use DAPI staining and scanning electron microscopy (SEM) evaluate the biofilm removal efficiency of anolyte and a combination of anolyte and catholyte.

LITERATURE REVIEW

1. Biocides

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

The use of biocides to control biofouling in water systems is still an accepted practice although higher levels of environmental awareness and tighter legislation have placed increased pressure on the water treatment industry to seek alternative means of control (6). Nevertheless biocides are still indispensable components for effective control of biofouling as they remain the core technology for decimating viable numbers whereas all other techniques merely aid in their efficacy.

The modes of action of a plethora of antibiotics have been investigated in detail. Much less is known about the mechanisms of action of the many biocides available for biofouling control. As antibiotics, biocides for water treatment target a range of specific cellular components and functions, from membrane permeability and electron transport to enzyme function.

Bactericides attack functional cell components, placing the bacterium under stress (52). At low concentrations bactericides often act bacteriostatically, and are only bacteriocidal at higher concentrations (58). Targets of bactericide action are components of the cytoplasmic membrane or of the cytoplasm (47). For bactericides to be effective, they must attain a sufficiently high concentration at the target site in order to exert their antibacterial action. In order to reach their target site(s), they must traverse the outer membrane of the gram negative bacteria. Therefore different bacteria react differently to bactericides due to differing permeabilities of their cell wall properties (40). Bacteria with effective penetration barriers to biocides generally display a higher inherent resistance than those bacteria which are readily penetrated. The rate of penetration is linked to concentration, so that a sufficiently high biocide concentration will kill bacteria with enhanced penetration barriers (47).

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

1.1 Oxidizing biocides

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

1.1.1 Peroxides

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

1.1.1.1 Hydrogen Peroxide

Silver enhances both the stability and antimicrobial effect of peroxide solutions. Peroxide has good antimicrobial properties and decomposes to water and oxygen, leaving no toxic waste. Hydrogen peroxide penetrates cells causing site-directed damage due to metal-dependant OH formation (1). It causes DNA strand breaks and base hydroxylation. Guanine and thymine are the two main targets of peroxide-generated free radical attack. The resulting 7,8-dihydro-8-oxoguanine mispairs with adenine whereas thymine oxidation products stop DNA polymerase, halting replication (1). Most bacterial mutants cannot survive due to incoherent metabolism, so that peroxide treatment at low concentration leads to slow death. Hydrogen peroxide also inhibits mitochondrial ADP-phosphorylation.

The development of resistance to oxidising bactericides has not been reported in the biofouling control literature. However, a variety of bacteria, mostly fermentative, exhibit oxidising stress response by producing oxidant-degrading and repair enzymes. Stress response means that cells become more resistant to a deleterious factor within hours of exposure to sub-inhibitory quantities of the factor. A variety of defense genes have been characterised in *Escherichia coli*, encoding various superoxide dismutases, catalyses, alkyl hydroperoxide reductases and glutathione reductases, as well as DNA repair enzymes (21). In addition various regulatory genes have been characterised, including *oxyR* and *soxR*. These regulators determine intracellular redox potential, and activate stress response when cells are exposed to oxidising agents.

1.1.1.2 Organic peroxides

Peracetic acid is the best known of the organic peroxides. Like hydrogen peroxide, it forms free hydroxyl radicals which react with various protein structures and DNA. In addition, the dissociation of peracetic acid leads to formation of acetic acid which is mildly antibacterial itself. Application of peracetic acid to systems does not leave any toxic waste behind. The antibacterial activity of peracetic acid is not affected by water hardness or organic contamination.

1.1.2 Oxidizing halogens

Hypochloric and hypobromic acids poses excellent antibacterial activity, although within a defined pH range.

1.1.2.1 Chlorine Compounds

Chlorine, chlorine dioxide and hypochlorous acid (HOCl) are the most widely used biocides worldwide. Hypochlorite was first employed as wound disinfectant by Hüter in 1831, and its bactericidal activity was confirmed by Koch in 1881 (53). Hypochlorite is used among others in industrial water systems to control biofouling.

The antibacterial mechanism of action of hypochlorite is not clear to date although much work on

the mechanism of action in eukaryotic cells has been done (49). HOCl is a powerful oxidising agent, oxidising thiol groups and halogenating amino groups in proteins (48), and oxidising lipids and proteins (28). Specific bacterial targets are cytochromes, nucleotides and iron-sulfur proteins (2). Protein synthesis is impaired (data not published) and the uptake of nutrients is also affected (4). The stability and antimicrobial activity of hypochloric acid is dependant on pH. It dissociates at pH greater than 7, and the undissociated moiety is the antibacterial one (53). Above pH 7,5 it loses its antibacterial activity. It is excellent for biofouling control as it weakens the extracellular polysaccharide (EPS) structure, leading to sloughing and removal of sections of the biofilm.

1.1.2.2 Bromine Compounds

Hypobromous acid works similarly to hypochloric acid. It is, however, stable at a pH up to pH 8.5. This makes it more suited for application in cooling waters which are often maintained at a slightly alkaline pH. Certain organic compounds release hypobromic and hypochloric acid slowly when in solution. An example is 3-bromo -1-chloro -5,5 –dimethylhydantoin (43). Such compounds maintain a longer antibacterial level of hypohalous acid in the system treated.

1.1.3   Ozone

Ozone is a strong oxidising material capable of killing bacteria and algae and of inactivating viruses. It is an unstable gas with a pungent odour. It further degrades the EPS holding biofilms together, so that treatment results in loosening of the biofilm. This leads to loosening of scale from the surface. Ozone has a very short half-life and therefore has to be generated on site. In distilled water its half-life at 20°C is 25 minutes. Its solubility in water is 13 times that of oxygen. Upon reaction with organic material it decomposes to oxygen. It does, however, react with several cations and anions such as $Fe^{2}+$, $Mn^{2}+$, MnO_4, NO^{2-}, and CN. Ozone is toxic to humans, and detectors should be installed together with ozone generators. However treated water is perfectly safe as ozone degenerates to oxygen.

1.1.4   Electrochemically activated water

1.1.4.1 Principles of ECA technology

Water of varying mineralisation is passed through an electrochemical cell, the specific design of which, permits the harnessing of two distinct and electrically opposite streams of activated water. Aside from its distinctive attributes, the negatively charged anti-oxidant solution (**Catholyte**) can also be channeled back into the anode chamber, thereby modulating the quality of the positively charged oxidant solution (**Anolyte**) that is produced. Depending on the specifications of the required application, variations in the design of the hydraulic systems can be effected to meet the requisite objectives.

The design of the cell is such, as to ensure a uniformly high voltage electrical field through which each micro-volume of water must pass. This unipolar electrochemical activation created by potential gradients of millions of volts per cm^2 between the anode and cathode terminals, results in the creation of solutions whose pH, Oxidation Reduction Potentials (ORP) and other physico-chemical properties, lie outside of the range which can be achieved by conventional chemical means.

1.1.4.2 Properties of Activated water

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

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

1. Stable products which include acids and bases which influence the pH of the solutions,
2. Highly active unstable products including free radicals and electrolytic gases in the form of micro-bubbles which influence the ORP of the solutions, and
3. Quasi-stable products comprising complexes of hydrated membranes which form clusters of water molecules which impart the catalytic activity of the solutions.

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

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

1.1.4.3 Biocidal properties of Anolyte

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

The advantages of the current ECA technology has been confirmed, wherein the biocidal activity of hypochlorous acid generated by the current ECA technology is 300 times more active than the Sodium hypochlorite generated by earlier systems. Additionally comparison of neutral Anolyte (pH=7), with alkaline Gluteraldehyde (pH=8.5), showed that the latter required a concentration of 2% versus 0.05% of the former, in order to achieve the same biocidal efficacy. Similarly, it has been shown that a 5% solution of sodium hypochlorite (Jik) can only be used for purposes of disinfection whilst a 0.03% solution of neutral Anolyte, has both disinfectant and sterilising properties. In general, the biocidal activity of non-activated neutral Anolyte (only stable products and no electrical charge) is 80 times the potential activity of the hypochlorite solution, but still exhibits only one third of the full biocidal potential of the optimally activated ECA solution. Thus, activated solutions have been conclusively shown to exceed chemically derived equivalents both in low dosage effectiveness as well as physico-chemical purity. This heightened biocidal capacity relative to traditional chemical solutions, permits the incorporation of ECA solutions at substantially lower dose rates, therein obviating the risk of intoxication, adverse environmental impact, while providing cost effective resolutions

1.2 Non-oxidising biocides

These include a variety of organic chemical compounds which have antimicrobial activity. Their modes of action differ vastly, and their only common denominator is that they are non-oxidising organic molecules. Most currently used biocides fall into five distinct categories, although a number of miscellaneous compounds are also usefull.

1.2.1 Detergent-type biocides

Three groups of surface-active antimicrobial agents have been documented to date; anionic, cationic, and amphoteric (53). Anionic antimicrobials are only effective at pH < 3.0 and include the aliphatic acids such as sodium dodecyl sulphate. The cationic antimicrobial agents are the generally organic ammonium salts, commonly termed quaternary ammonium compounds. The best known is benzalkonium chloride (N-alkyl-N,N-dimethyl benzylammonium chloride) (53). Recently a number of novel organic ammonium salts of general structure alkyl trimethyl – and dialkyl dimethyl ammonium bromide were synthesized and evaluated (37). The authors reported extremely low minimum inhibitory concentrations to *P. aeruginosa* of 12.5 µg/ml where the second alkyl group was C_6 or longer.

Benzalkonium chloride adsorbs to the cell surface of negatively charged cells (pH > 7.0) in an irreversible way (47). The pH minimum for antimicrobial activity is 3.0. It is membrane active and induces leakage of cytoplasmic constituents (53). Upon exposure to benzalkonium chloride, membranes of *P. cepacia* appeared irregular, indicating membrane damage (45). At 37 °C it is twice as active as at 20 °C. It is active against gram positive and also against gram negative cells, but not against spores. Cations such as Ca^2+ and Fe^3+ decrease its activity, as does NaCl (53).

1.2.2 Biguanides

Biguanides are polymer derivatives of a general guanidine structure. Two biguanides are currently used as industrial bactericides. These are polyhexamethylene biguanide (PHMB) and 1,6-di-(4-chlorophenyldiguanido)-hexane, better known as chlorhexidine (53). Both are not corrosive and all are well suited for application in cooling water (58). Biguanides are bacteriostatic at low concentrations and bactericidal at higher concentrations, and have a wide spectrum of activity, especially against gram negative bacteria (53). They are membrane active agents and attach rapidly to negatively charged cell surfaces (pH neutral or alkaline). By making use of ^{14}C-radiolabelled PHMB, it has been shown that PHMB 15 absorbed into cells of *E. coli* within 20 s after exposure (22). Bactericidal action, however, requires a few minutes. Biguanides compete with divalent cations for negative sites at LPS, displacing these. PHMB then interacts by elecrostatic interactions with the charged headgroups of phosphatidylglycerol and diphosphatidylglycerol (negative), but not with the neutral phosphatidylethanolamine (8). By binding to phospholipids of the inner leaflet of the outer membrane and of the outer leaflet of the inner membrane, the two membranes attain net positive charges and are repelled from each other, causing membrane damage by distortion. This is supported by TEM studies on *P. cepacia* where both membranes acquired a distinct irregular appearance after treatment with chlorhexidine (45). Cytoplasmic constituents start leaking out of the cell due to rupturing of the membranes, and the cell loses its viability.

1.2.3 Aldehyde-based biocides

Two aldehydes are commonly used as antimicrobial agents, ie. formaldehyde and gluteraldehyde. Further there is a range of bactericides such as the hydroxyethyl- and ethyltriazine- bactericides available which all release formaldehyde (46). Formaldehyde has a high polarity and high nucleophilic reactivity, so that it reacts primarily with free primary amino groups, but also with amines, amides, sulfides, purines and pyrimidines (46). In water it hydrates to methylene glycol. Reaction with primary amino groups leads to the formation of methyloamines which react further with cellular components. Formaldehyde damages the transport properties of membrane porins, decreasing the rate of proline uptake and of enzyme synthesis. It is active over a wide pH spectrum (3.0-10.0), and is sporicidal (53).

Gluteraldehyde also reacts with amino and sulfhydryl groups (47). It is stable in acid solution but is only active at pH 7.5 - 8.5, so it must be alkalinified before application (53). A 2 % solution at the correct pH is ten times more bactericidal than a 4 % solution of formaldehyde (53). Its reactivity is related to temperature; a 2 % solution kills spores of *Bacillus anthracis* in 15 min at 20 °C, whereas it requires only 2 min at 40 °C. In gram positive bacteria it reacts with, and binds to, peptidoglycan and teichoic acid, and is also sporicidal (47). In gram negative bacteria it reacts primarily with lipoproteins of the outer membrane, preventing the release of membrane-bound enzymes.

1.2.4 Phenol derivatives

Phenol was the antimicrobial agent which revolutionised invasive surgery, and was pioneered by Lister in 1870 (23). It enters the cell by dissolving in the membrane, and upon entry into the cytoplasm, precipitates proteins. It is, however, harmfull to humans, and its antibacterial activity is not very high (53). A range of halogenated phenols, cresols, diphenyls and bisphenols have been developed from phenol, and have excellent antimicrobial activity, many being applied in the preservation of pharmaceutical products. Halogenation increases the antimicrobial activity of phenol, as does the addition of aliphatic and aromatic groups (53). Bisphenols have the highest antimicrobial activity of the phenol derivatives, especially halogen substituted ones. Hexachlorophen and 2,2'-methylenebis(4-chlorophenol) (dichlorophen) fall into this group (11).

Phenol derivatives are membrane active agents. They penetrate into the lipid phase of the cytoplasmic membrane, inducing leakage of cytoplasmic constituents (47). 3- and 4-chlorophenol uncouple oxidative phosphorylation from respiration by increasing the permeability of the cytoplasmic membrane to protons (24).

1.2.5 Thiol-oxidizing biocides

Thiols on amino acids such as cysteine are important groups which influence the tertiary structure of proteins by the forming disulphide bridges. Three groups of antimicrobial agents, isothiazolones, Bronopol (2-bromo-2-nitropropane-1,3-diol), and mercury and other heavy-metal compounds, react with accessible thiols, altering the three dimensional structure of enzymes and structural proteins (15). Mercury interacts with sulfhydryl groups by complexing with sulphur (53). Bronopol oxidises thiols to disulphides, reacting especially with the active center of hydrogenase enzymes (53).

Four water-soluble isothiazolones possess antibacterial activity; 5-chloro-2-methyl-3-(2H)-isothiazolinone (CMIT), 2-methyl-3-(2H)-isothiazolinone (MIT), 1,2-benzisothiazolin-3-one (BIT) and 2 methyl 4,5-trimethylene-4-isothiazolin-3-one (MTI) (53). MIT and CMIT are often supplied in a 3:1 ratio. Isothiazolones react oxidatively with accessible thiols such as cysteine and glutathione (16). These thiols are oxidised to their disulphide adjuncts which, in the case of cysteine, leads to an alteration of protein conformation and functionality. Isothiazolone is hereby reduced to mercaptoacrylamide, which in the case of CMIT tautomerises to thioacyl chloride, the latter reacting with amines such as histidine and valine (15). Isothiazolones are primarily bacteriostatic, and are only bactericidal at high concentrations.

1.2.6 Miscellaneous biocides

The mechanisms of action of various antimicrobial agents, employed to control bacterial growth in cooling water systems, have not been formally published to date. These include tetra-alkyl phosphonium chloride, Na diethyldithiocarbamate, methylene bisthiocyanate (MBT), 2-thiocyanomethylthiobenzothiazole, 2-thiocyanomethylthiobenzothiazole, hexachlorodimethyl-sulphone, tetrakishydroxymethylphosphonium sulphate (THPS) and a range of 2-arylthio-N-alkylmaleimides (29).

Phosphonium chloride probably has surfactant properties, damaging the bacterial cell envelope. It is active over an extremely wide pH range (2-12) and can be used in conjunction with oxidising biocides. MBT is readily miscible with dispersants to aid with penetration into and dispersion of biofilms. The mode of action of this non metallic organosulphur compound is unknown to date. Thiocarbamates are used as agents for the extraction of trace metals such as Fe, Cd, Co, Cu, Mn, Ni, Pb and Zn (34). This would imply that it chelates iron, a vital trace element of most bacteria. The nucleophilic sulphur atom indicates potential reactivity with accesible thiols. Thiocarbamates do react with accessible thiols and amines. Therefore their antibacterial mechanism of action would rest partially on denaturation of surface proteins. We have found that the antibacterial mechanism of action depends on the alkyl chain length of the thiocarbamate. Sodium die-thyldithiocarbamate is inactivated by free ferrous iron, indicating that it removes iron (a growth factor) from the cell. Sodium dimethyl dithiocarbamate is not inactivated to the same degree, showing that its antibacterial activity does not rest as much on iron removal. 2-Thiocyanomethylthio-benzothiazole is also stable over a wide pH range and can be used synergistically with surfactants to aid in dispersion of and / or penetration into biofilms. Hexachlorodimethylsulphone is stable at high temperature and is an effective slimicide over a wide pH range, although it is not water soluble and must be dosed as an emulsion. THPS is an amphoteric surfactant miscible with various surfactants to improve action against biofilms. It is active in both acidic and alkaline conditions and at high temperatures, and remains water soluble in oil-water mixtures.

The 2-arylthio-N-alkylmaleimides are extremely bacteriostatic, and many derivatives demonstrate an MIC against various bacteria except *P. aeruginosa* of 6.25 μg/ml (29).

1.3 Alternative approaches of chemical control

1.3.1 Surface-embedded biocides and catalysts
Various tertiary amines have been covalently linked to polystyrene (20), and quaternary amine compounds have been complexed into polyvinyl chloride and ethylene vinyl acetate latex (44). The broad-spectrum antimicrobial Triclosan (2,4,4'-trichloro-2'-hydroxy-diphenylether-5-chloro-2-(2,4-dichlorophenoxy)-phenol) has been impregnated into various polymers and shown to suppress surface-associated growth. It is active against most bacteria, excepting *P. aeruginosa*. Recently (57) reported a strategy for circumventing the resistance of bacteria in biofilms by generating biocidal species at the biofilm-substratum interface from less active agents using surface-embedded catalysts. They embedded the catalysts cobalt- and copper sulphonated phthalocyanine in surface-associated resin. Both hydrogen peroxide and potassium monopersulphate are broken down to highly reactive oxygen species and significantly enhanced the killing of *Pseudomonas aeruginosa* biofilms (56).

1.3.2 Enhancement of biocidal activity by an electrical field
Bacteria in biofilms are well known to be more resistant to biocides than are their planktonic counterparts (see section 3.3). A select number of studies have reported that application of low-intensity electric fields in the range 15 μA/cm^2 to 2.1 mA/cm^2, and with a field strength of 1.5 to 20 V/cm can override the biofilm –associated resistance (5, 17). All areas within the electric field are affected as the antimicrobial agent is moved by an electrophoretic effect.

1.3.3 Saccharolytic enzymes
The biofilm is held together by an extracellular polysaccharide. The extracellular polysaccharides are composed of homo- and heteropolysaccharides of mannose, glucose, fucose, galactose, mannuronic acid, guluronic acid and pyruvate, giving rise to a complex array of polymeric structures (7). Saccharolytic enzymes have been used to degrade the polysaccharide matrix, thereby destabilising the biofilm structure, but the heterogeneity of polymers dictates that mixtures be applied (7, 50). Recently (31) demonstrated that the addition of lactoperoxidase to saccharolytic cocktails contributed not only to biofilm removal, but also to a decrease in the culturable count.

1.3.4 Dispersants (surface active compounds)
More recently surface active compounds (surfactants) have been employed to prevent bacterial adhesion to surfaces. It is unlikely that surfactants will have any mutagenic effects on bacteria, or that microorganisms would be able to become resistant to the action of surfactants, as can be the case with biocides (47, 10). Unfortunately little published information is available on the effectivity of different biodispersants (surfactants) against bacterial attachment (35). According to (42), dilute surfactants completely inhibited the attachment of estuarine and marine bacteria. Surfactants result in both uniform wetting of the surface to be treated and have an additional cleaning effect (10, 35). Whittekettle (54) found a correlation between the ability of a surface-active compound to lower surface tension and its ability to prevent microbial adhesion. White and Russel (55) classified surfactants according to the ionic nature of the hydrophilic group viz. anionic, cationic, non-ionic and zwitterionic.

2.   Factors affecting efficacy of treatment programmes

The antibacterial activity of bactericides is determined by their chemical reactivity with certain organic groups. Bactericides do not select between free and cell-bound groups. Therefore oxidising bactericides react with any readily oxidisable organic compound, and not only with live cells. Bactericide activity is influenced by the chemistry of the surrounding where it is employed (53) . Factors affecting bactericide effectivity are the following:

- pH
- water hardness
- organic compounds such as proteins or saccharides
- additives such as antiscaling agents or corrosion inhibitors

These factors affect different bactericides to different degrees. Some bactericides are not very stable in concentrated form and undergo changes. Formaldehyde polymerises when exposed to polar compounds (acids or alkalis) or high temperature and oxidises to formic acid when exposed to air (53). Isothiazolones are unstable at temperatures above 40 °C and chlorhexidine is unstable above 70 °C (53). A decrease in the efficacy of a bactericide treatment programme can be due to a decrease in bactericide activity, or due to inactivation by adverse conditions, and does not always indicate bacterial resistance (13).

2.1   Chemistry of the water

Chemicals inhibiting scaling and corrosion are also added to industrial water systems, and some interact with certain biocides. Chromates are used to inhibit corrosion, but also suppress microbial growth, acting synergistically with the biocide used. Glycolic acid secreted by algae can, however, reduce chromate, rendering it inactive. Dithiocarbamates reduce chromate, so the two substances are incompatible (34). QAC's form insoluble chromate precipitates at high concentrations, so the two should not be added simultaneously to water. Careless application of chloride lowers the pH to a point where the protective chromate film is solubilised. Na-2-mercaptobenzothiazole is a corrosion inhibitor which is oxidised by chlorine dioxide. Methylene bis-thiocyanate is hydrolysed under slightly alkaline conditions (pH 7,5). Where the chlorine demand of the water is high, the large quantity of chlorine added leads to a high chloride level which increases the corrosion potential of the water. Chlorine and quaternary ammonium compounds increase the corrosion rate of copper alloys.

2.2   Bacterial resistance

Bactericide treatment regimes for cooling water systems often fail, posing the question of bacterial resistance to the bactericide. Certain authors have argued that failure of treatment programmes was due to selection for resistant strains. We have however shown that susceptible bacterial isolates do acquire increased tolerance to bactericides following serial transfer in sub-inhibitory concentrations. Resistance has been defined as the temporary or permanent ability of an organism and its progeny to remain viable and / or multiply under conditions that would destroy or inhibit other members of the strain. Bacteria may be defined as resistant when they were not susceptible to a concentration of antibacterial agent used in practice. Traditionally, resistance refers to instances where the basis of increased tollerance is a genetic change, and

where the biochemical basis is known. Whereas the basis of bacterial resistance to antibiotics is well known, that of resistance to antiseptics, disinfectants and food preservatives is less well understood. The basis of bacterial development of resistance to water treatment bactericides is little known.

As biocides are selective in their action, application of any one could result in selection for resistant bacteria. As cells in biofilms and planktonic communities are in continuous exchange, death of cells in the planktonic phase would influence the equilibrium and shifts would occur in both the planktonic and the sessile populations. Biocides attack targets of cell function, placing the bacterium under stress. It is well recognized that communities under stress have a lower species diversity and select for fitter species. Therefore a more resistant community could develop. The concentration of a biocide is not related linearly to its activity; a concentration exponent is involved in the relationship. In many cases a small decrease in concentration will result in a notable decrease in activity.

In an *in situ* biocide evaluation study we found the dominant planktonic survivor after 48h was a species most effectively killed by the relevant biocide under laboratory pure culture conditions. An example is dichlorophen which killed 99.94% *ofPseudomonas stutreri* at 50 ppm and yet left this species the dominant isolate after 48h (43%) in the cooling system. Also, thiocarbamate killed 99.87% of *P. stutzeri* at 174ppm and left it the dominant planktonic survivor (62.5%) in the system treated. QAC-tin killed 100% of *Acinetobacter calcoaceticus* and left *Acinetobacter* spp. dominant (40%)in the system. Although the surviving strains could be different ones, the correlation is striking.

Two reasons exist why the efficacy of bactericide treatment programmes can decrease at times. The one is a decrease in the activity of the bactericide, and the other is a decrease in the bacterial susceptibility towards the bactericide. Three mechanisms of resistance have been reported in the field of antibiotic study:
*Inaccessibility of the antimicrobial agent to its site of action,
*Absence of the susceptible site, or alteration to an insusceptible form, and
*Inactivation of the antibacterial agent.

Bactericides are less specific in their action than some antibiotics, so that the alteration of a reactive site or the substitution of an amino acid in a protein will not render bacterial cells resistant. Therefore inaccessibility and inactivation are the two classes of possible mechanisms of resistance. Additionally, active removal of biocide is a third class of resistance.

2.2.1 Decreased permeability
The initial stage of bactericide action is binding to the bacterial cell surface. Then it must traverse the cell wall (gram positive) or outer membrane (gram negative) to reach its site of action at the cytoplasmic membrane or cytoplasm. In gram positive bacteria there are no specific receptor molecules or permeases to assist or block bactericide penetration. The cell wall of *Bacillus megaterium* is permeable to molecules up to 30 kI). Intrinsic resistance of gram positive bacteria to bactericides is therefore low. The gram negative cell envelope has, however, evolved to regulate the passage of substances into and out of the cell to a remarkable degree of specificity.

All the components of the cell envelope except peptidoglycan play a role in the barrier

mechanisms because peptidoglycan is spongy and therefore permeable. *P. aeruginosa* is the most resistant non-sporeforming bacteria to most bactericides, due to the superior barrier properties of its outer membrane. In a recent study the antimicrobial activity of a series of new 2-arylthio-N-alkylmaleimides were compared and many were found active against *Staphylococcus aureus*, *Bacillus subtilis* and *E. coli*. Only one of the 51 derivatives tested was marginally active against *P. aeruginosa*.

The physiological state of cells and the nature of the habitat can lead to considerable variation in the susceptibility of bacteria to bactericides. The composition of the bacterial cell envelope does change as a response to available or limiting nutrients, so that the barrier properties of the envelope are affected. Exposure to sub-inhibitory concentrations of bactericides can lead to phenotypic adaptation, resulting in a resistant cell population. In *E. coli* certain proteins induced by heat or starvation stress also confer resistance to 11202 and to UV light. Most bactericide-resistance is due to adaptation, and the resistant phenotype is mostly lost upon removal of the bactericide.

2.2.2 Efflux systems

Bacteria can actively pump compounds out of the cell via membrane efflux systems. Only one type of bactericide-efflux system has been described to date, the QAC efflux system of *Staphylococcus aureus*. This efflux system is coded by two gene systems. The genes *qacA* and *qacB* encode for a high level of resistance, and *qacC* and *qacD* encode for a low level of resistance. *qacC* and *qacD* are further identical to the *ebr* gene encoding for resistance to ethidium bromide in £ *aureus*, explaining why resistance to QAC is often concurrent with resistance to ethidium bromide. The *qacA* gene codes for a 50 kD protein which mediates energy-dependant efflux of both benzalkonium chloride and ethidium bromide. The *qacc* gene also mediates energy-dependant efflux of benzalkonium chloride and ethidium bromide. Two different but isofunctional gene systems appear to have evolved in S *aureus*.

2.2.3 Enzymatic degradation of biocides

Resistance to antimicrobial agents can be due to enzymes transforming the bactericide to non-toxic form. The phenomenon is usually investigated from the biodegradation point of view, *ie.* the biodegradation of toxic pollutants. A host of aromatic, phenolic and other compounds toxic to many bacteria (some of which are employed as bactericides) can be degraded by certain bacteria. The topic has been reviewed by various authors and the current literature reports extensively on biodegradative pathways.

Two types of enzyme-mediated resistance mechanims have been documented, *ie.* heavy metal resistance and formaldehyde resistance. Resistance to heavy metals includes resistance to the following: mercury, antimony, nickel, cadmium, arsenate, cobalt, zinc, lead, tellurite, copper, chromate and silver. Detoxification is usually by enzymatic reduction of the cation to the metal. Where some heavy metal resistance genes are carried on plasmids, others are chromosomal. The resistant phenotype is usually inducible by the presence of the heavy metal. Some heavy metals induce resistance to a broader spectrum of heavy metals. Arsenate, arsenite and antimony, for example, induce resistance to each other in *E. coli*.

The detoxification of formaldehyde by P. *aeruginosa and P. putida* has been studied extensively.

Formaldehyde is reduced by an NAD + - gluthathione-dependant dehydrogenase, formaldehyde NAD + oxidoreductase. This enzyme is probably plasmid-encoded, and appears to be constitutively expressed. Resistance to most formaldehyde-releasing formaldehyde condensates is also due to formaldehyde dehydrogenase activity as the antibacterial mechanism of these condensates appears to be via formaldehyde.

2.3 Biofilm-associated resistance

Bacteria in biofilms are much more protected from bactericidal action than are planktonic bacteria. In a recent study, biofilm bacteria were found 150 to 3000-fold more resistant to hypochlorous acid and 2 to 100 times more to monochloramine than were unattached cells. *Pseudomonas aeruginosa* growing as a biofilm has been found 20 times as resistant to tobramycin as are planktonic cells. Three reasons for the increased biocide resistance of biofilm bacteria have been put forward. These do not adequately explain the phenomenon of biofilm resistance, but they are listed below:

1.   The EPS material is a polyanionic polymer and acts as an exchange resin. It quantitatively adsorbs biocide, protecting the bacterial cell from biocide action. Biofilms contain large amounts of EPS which would protect resident bacteria from biocides.
2.   Gram negative bacteria growing in biofilms have a higher ratio of unsaturated to saturated fatty acids and a higher ratio of C_{16} to C_{18} fatty acids. Resistant bacteria show similar changes in membrane-lipid profiles.
3.   Surface hydrophobicity of QAC and amphoteric resistant cells was higher than that of unadapted cells. Biofilm bacteria often have a higher surface hydrophobicity due to attachment structures.

The first reason is questionable because the reason why bacteria grow in biofilins is because the EPS acts as a nutrient sink, attracting organic compounds from the surrounding water. Recent work has supported the theory that organic material is somehow attracted to the EPS, and associates favourably with it. At least some of these molecules must diffuse to and into the microorganisms embedded in the EPS to facilitate the observed growth. Non-oxidising biocides, being organic molecules of small to intermadiate size would also associate favourably with the EPS. At least some would diffuse to and into the microorganisms embedded in theEPS and exert their antibav\cterial activity. The mechanism of increased resistance must be related to alterred surface properties of cells growing in the biofilm environment. Reasons 2 and 3 are possibly correct.

2.4 Mutagenicity of biocides

Some bactericides, being toxic substances, have a broader spectrum of toxicity than merely bacteria. Some are mutagens or even carcinogenic. Table 4 summarises the LD_{50} values available for bactericides, as well as mutagenicity data where available.

Mutagens are substances which induce mutations in DNA of any living organisms. Furthermore many mutagens are carcinogens. Mutation in bacteria from water cooling systems will increase the rate of development of resistance to biocide, and necessitate the addition of higher levels of biocide. Such treated water, when released into the environment will cary with it large volumes of mutagenic and possibly carcinogenic biocides. This will affect ground water sources, rivers, dams

and by irrigation agricultural soils. It could also reach drinking water sources. The release of such substances into nature is dangerous to human life.

Formaldehyde itself is carcinogenic. A second mutagenic compound used in biocides is 5-chloro-N-methylisothiazolone (CMIT). The related isothiazolones benzisothiazolone (BIT) and N-methylisothiazolone (MIT) are not mutagenic. Thiocarbamates are not toxic to humans as they are degradation products of certain pharmaceuticals used in the treatment of alcoholism.

MATERIALS AND METHODS

Organism used
Pseudomonas aeruginosa isolated from a cooling water system was used for all the experiments (10).

Biocide used
Anolyte and Catholyte was supplied by Radical waters. In the first experiment anolyte was used in a 1:10 dilution. In the second experiment a combination of anolyte and catholyte at a ratio of 2:1 was used in a 1:10 dilution.

Experimental procedures
A continuous flow - through system Pedersen device (39) was used to determine the biofilm removal of *Ps. aeruginosa* on a stainless steel surface and on glass.

DAPI-staining
DAPI staining was done as described in a previous study (Wolfaardt et al., 1996). Qantification of attached bacteria using 4,6-diamidino-z-phenylidole (DAPI). The 75 x 27 x 1mm coupons, were removed from the Pedersen device and rinsed with sterile water as described for the SEM studies of biofilm formation and stained with DAPI for epifluorescence microscopy (Wolfaardt et al., 1991). Attached bacteria were observed under oil immersion using Epifluorescence microscopy. Ten randomly chosen microscope fields were counted under the 800 x magnification.

Scanning Electron Microscopy (SEM)
Coupons (25 x 27 x 1mm) were removed, in duplicate, from the modified Pedersen device at 4, 8, 24, 32, 48 and 56 h, with a sterile forceps and replaced with a sterile coupon, in order to keep the flow constant. After removal the coupons were rinsed with sterile distilled water for 30 s to remove any unattached cells and then fixed for SEM by the following series of treatments: 2% gluteraldehyde (1 h); 0.175M Phosphate- buffer (3 x 15min); 50% ethanol (1 x 15min); 70% ethanol (1 x 15min); 90% ethanol (1 x 15 min) and 100% ethanol (3 x 15min). The coupons were thereafter dried in a critical point dryer, mounted on studs and coated with gold plasma and examined using the Hitachi S-450 scanning electron microscope.

Biofilm removal
To study biofilm removal, the bacteria were allowed to adhere to the surface of the 3CR12 stainless steel coupons for 168h in R2A agar, before the dosing of the biocide. The experiment

was allowed to proceed for 78 h. Samples were removed before treatment and hourly for 6h following treatment. Biofilm removal was determined with DAPI and SEM.

Anolyte was added to the system at a 1:10 ratio for the first experiment. A mixture of anolyte/catholyte (2:1) was added at a 1:10 ratio in the second experiment.

For both experiments, a control system, using dam water, where no biocide was added, was included.

Viable bacteria counts

The total number of viable bacteria in the planktonic phase was determined before biocide addition and again after 6h. Plate counts were done on R_2A agar and incubation at ambient temperature to simulate the experimental conditions.

RESULTS AND DISCUSSION

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

Table 1   Planktonic bacterial numbers before and after anolyte treatment

Time	cfu ml^{-1}
Before addition	$2,41 \times 10^7$
After 6h	<10
After 24h	<10
After 72h	$1,33 \times 10^6$

Cfu = colony forming units

The anolyte/catholyte (2:1 ratio) solution added at a 1:10 ratio also effectively removed the mature *P.aeruginosa* biofilm. The anolyte/catholyte solution effectively removed the biofilm within 3-4h (Fig 2). Noticeable is the dispersion of the biofilm structure (after 1h) before removal occurs (Fig 2). Regrowth of the biofilm started taking place 24h after

treatment (Fig 2). Regrowth of the planktonic bacteria occurred after 72h (Table 2).

Table 2 Planktonic bacterial numbers before and after anolyte/catholyte treatment

Time	cfu ml^{-1}
Before addition	1,14 x 10^7
After 6h	< 10
After 24h	< 10
After 72h	1,5 x 10^6

Cfu = colony forming units

Fig's 3-9 are representative scanning electron micrographs of the biofilm behaviour before and after biocide treatment. Figure 3 and 4 represent the biofilm before and after 1h of treatment. Surface colonization can clearly be seen by numerous microcolonies. Also noticeable is the dehydrated glycocalyx structure (biofilm). These micrcolonies are still visible after 2h (Fig 5) and 3h (Fig 6) of treatment. The microcolonies are nevertheless fewer in number and smaller in size than at 0h and 1h. After 4h of treatment (Fig 7) very few microcolonies were observed and the glycolacyx (biofilm) was no longer noticeable. After 24h of treatment the situation remained unchanged (Fig 8). Neverthless, DAPI staining indicated regrowth of the biofilm (Fig 2). This difference was attributed to the difference in the method of preparation for DAPI and SEM, where the preparation of slides for DAPI is less harsh than for SEM.

CONCLUSIONS

- Both the anolyte and the anolyte/catholyte combination effectively removed a mature *P. aeruginosa* biofilm and reduced the bacterial numbers from > 10^7 cfu ml^{-1} to less than 10 cfu ml^{-1} within 6h.

- Regrowth of the biofilm was observed 24 h after biocide treatment as was also the case for the planktonic bacteria numbers.

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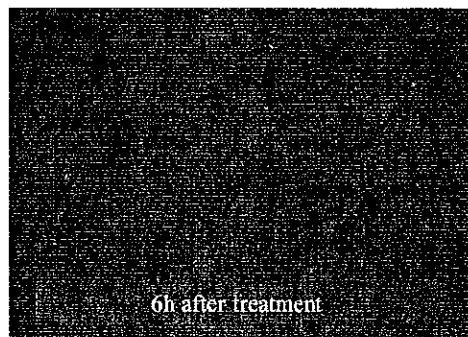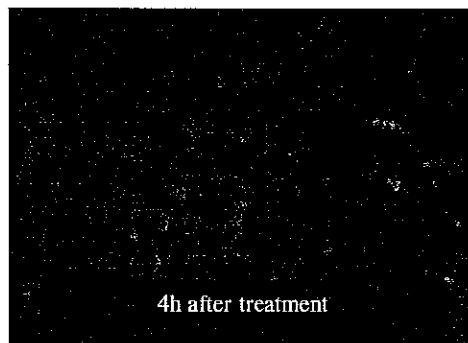
Control

Before treatment with

1h after treatment

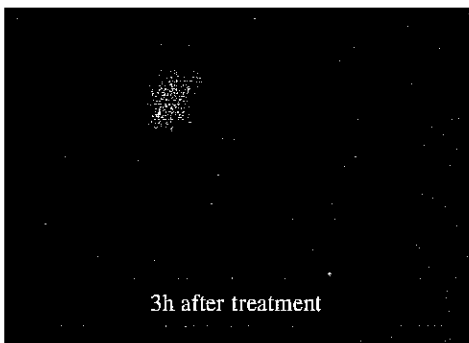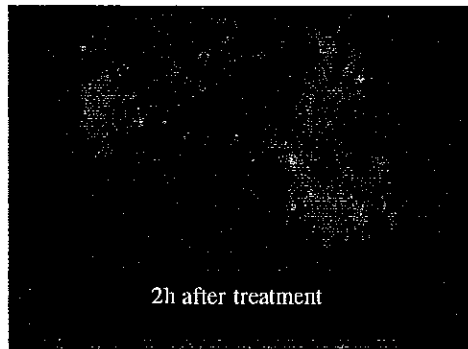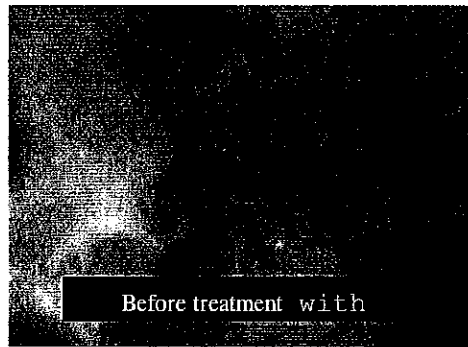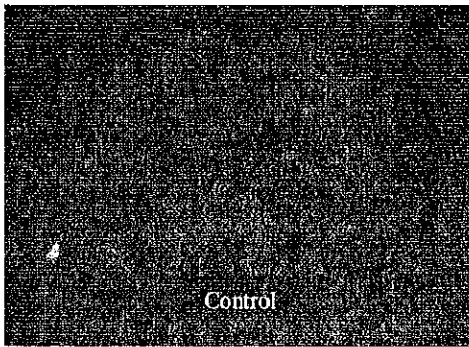2h after treatment

3h after treatment

4h after treatment

5h after treatment

6h after treatment

Figure 1. Biofilm removal using anolyte

24h after treatment

72h after treatment

Before treatment with Anolyte
Catholyte combination

1h after A-C treatment

2h after A-C treatment

3h after A-C treatment

4h after A-C treatment

24h after A-C treatment
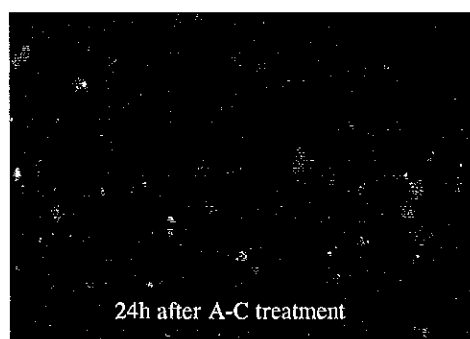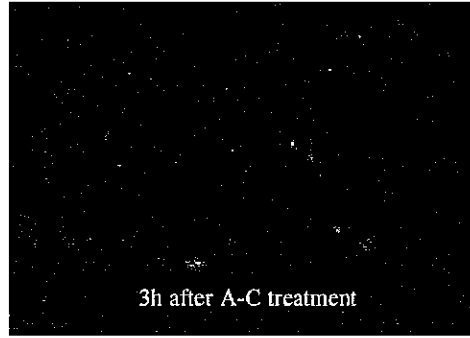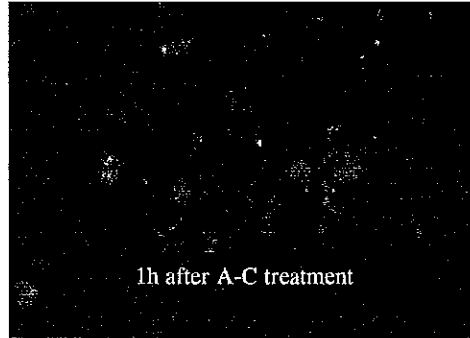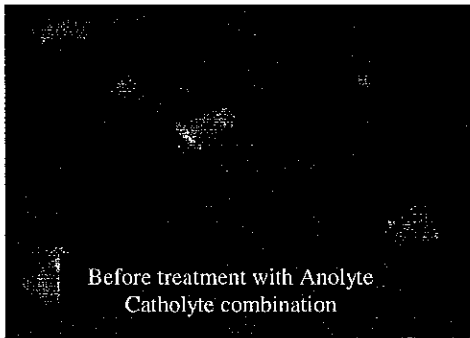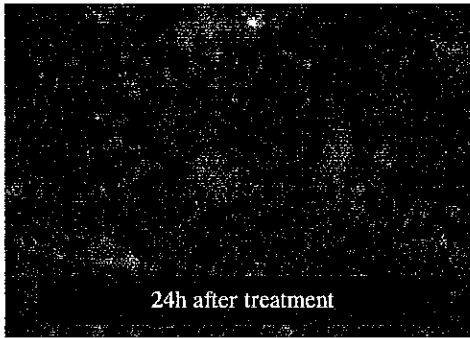
Figure 2 Regrowth of biofilm after treatment with anolyte and biofilm removal
after treatment with the anolyte/catholyte (A-C) combination
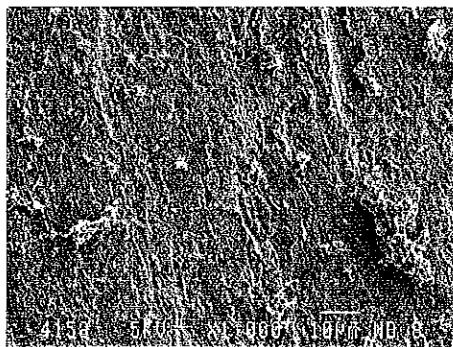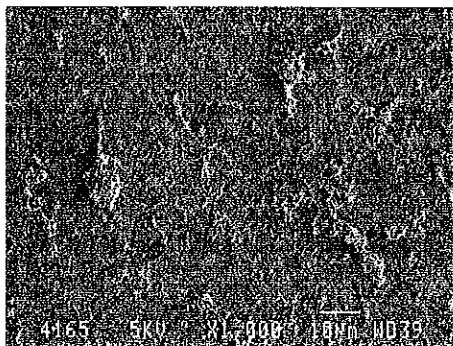
Figure 3. Biofilm before treatment
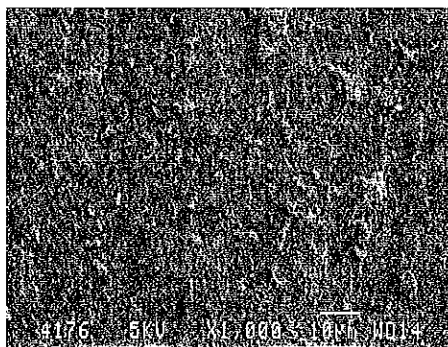
Figure 4. Biofilm after 1h of biocide treatment

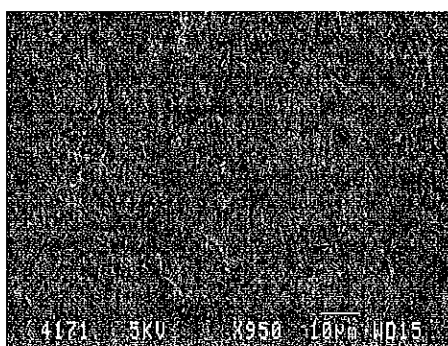Figure 5. Biofilm after 2h of biocide treatment

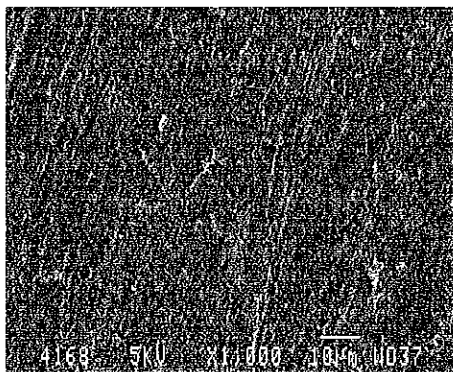Figure 6. Biofilm after 3h of biocide treatment
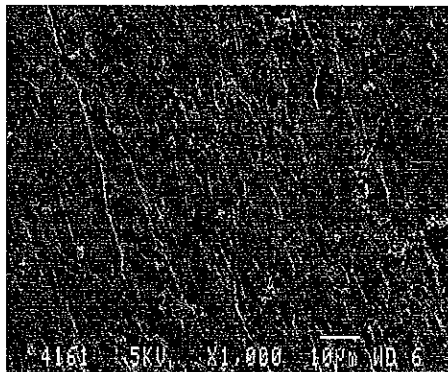

Figure 7. Biofilm after 4h of biocide treatment

Figure 8. Biofilm after 24h of biocide treatment