Differential response of sessile and planktonic bacterial populations following exposure to antimicrobial treatment

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

SUMMARY

The ability of biofilms to resist antimicrobial treatment, when planktonic microbes cannot, is of not only fundamental scientific interest, but also a concern in industrial and medical fields. The inability to control biofouling of water distribution networks and products, as well as recurrent infections of implanted medical devices, is not only costly, but also potentially lethal. Several mechanisms whereby biofilms are able to evade antibiotic and biocidal agents have been proposed and investigated, but no universally relevant characteristic has been identified.

Initial investigation, involving *Bac*Light™ LIVE/DEAD viability probes, epifluorescence microscopy and image analysis into the ability of natural biofilm and planktonic populations, cultured *in situ* in a cooling tower, to survive treatment with a commercial biocide was not conclusive. Subsequent laboratory experimentation with a bacterial isolate from the cooling tower water revealed that the ability of attached biofilms to resist antimicrobial treatment exceeded that of planktonic cells shed from the biofilm. The reduced ability of suspended cells to survive antimicrobial treatment was not statistically significant, compared to that of the biofilm ($P = 0.05$). This is in contrast to the wealth of literature published on the subject of biofilm antimicrobial resistance.

The dilution rate in the flowcells in which biofilms were cultivated was more than 100 times higher than the maximum specific growth rate of the test organism. Nevertheless, there was typically more than 1×10^8 cells/ml in the effluent, suggesting that a metabolically active, rapidly dividing layer of cells existed at the biofilm bulk-liquid interface, from where daughter cells continuously detached. Treatment with an antimicrobial agent resulted in a significant reduction in the viability and number of cells detached from the biofilm, suggesting that this metabolically active layer of the biofilm was more sensitive to antimicrobial treatment, possibly due to a higher specific growth rate. Antimicrobial resistance was shown to be affected by the growth rate for planktonic bacterial populations, with an increased ability to survive, correlated with a decrease in specific growth rate. This supports the contention that growth rate plays a role in the susceptibility of the active layer. The bacterial cells in the layers closest to the attachment surface of the biofilm has frequently been shown to be slow growing, due to nutrient and oxygen limitation, while the outer biofilm layer is more susceptible to unfavourable environmental conditions. It is possible that such differentiation, which results in a responsive outer biofilm layer, provides a mechanism for the protection of the cells in the deeper layers, and thus survival over time.

The results presented here support several hypotheses put forth in literature to account for the increased resistance of biofilms towards antimicrobial agents. Future work will include an investigation into changes in the patterns of gene expression when a bacteria becomes attached to a surface, upon subsequent release from the biofilm, and the influence this has on the ability to resist antimicrobial treatment.

OPSOMMING

Die vermoë van aangehegte mikrobes, in teenstelling met vrydrywende mikroorganismes, om behandeling met antimikrobiese middels te oorleef, is nie net van belang vanuit 'n fundamenteel wetenskaplike oogpunt nie, maar ook betekenisvol vir die industriële en mediese velde. Die beheer van bio-bevuiling van waterverspreidingsnetwerke en produkte, sowel as herhaalde infeksies van mediese inplantings, is nie net van kostebelang nie, maar ook potensieël lewensgevaarlik. Verskeie meganismes wat biofilms in staat stel om antimikrobiese behandeling te oorleef, is voorgestel en ondersoek, maar geen alomteenwoordige eienskap is tot dusver geïdentifiseer nie.

Aanvanklike ondersoeke na die vermoë van natuurlike biofilms en planktoniese gemeenskappe, om biosiedbehandeling *in situ* in 'n lugversorgingskoeltoring se water te oorleef, was onbeslis. Die eksperimentele metodes het gebruik gemaak van *Bac*Light™ LIVE/DEAD lewensvatbaarheidkleurstof, epifluoressensie-mikroskopie en beeldanalise. Daaropvolgende ondersoeke met 'n bakteriese isolaat vanuit die koeltoring het daarop gedui dat biofilms beter in staat is om antimikrobiese behandeling te oorleef as selle wat vrygelaat word vanuit die biofilm. Die afname in the lewensvatbaarheid van vrydrywende selle, na afloop van biosiedbehandeling, was nie statisties beduidend in vergelyking met die van die biofilm nie ($P = 0.05$). Die bevinding is in teenstelling met wat algemeen aanvaar word in die literatuur.

Die verdunningstempo waaronder die biofilms in die vloeiselle gekweek is, was meer as 100-voudig hoër as die maksimum spesifieke groeitempo van die toetsorganisme. Ten spyte hiervan was daar tipies meer as 1×10^8 selle/ml in die uitvloeisel teenwoordig. Dit dui op 'n metabolies aktiewe, vinnig verdelende laag selle in die boonste laag van die biofilm, naaste aan die vloeistof fase, waarvandaan dogterselle voortdurend vrygestel word. Behandeling met die antimikrobiese agent het 'n beduidende afname in die lewensvatbaarheid en aantal dogterselle tot gevolg gehad, wat lei tot die gevolgtrekking dat die metabolies aktiewe laag van die biofilm meer sensitief is vir antimikrobiese behandeling, moontlik weens 'n hoër spesifieke groeitempo. Daar is verder bewys dat die vermoë om die werking van die antimikrobiese middel teen te staan, afhanklik is van die spesifieke groeitempo van planktoniese populasies. 'n Afname in groeitempo word geassosieer met 'n toename in oorlewing na antimikrobiese behandeling, wat die voorstel dat die groeitempo van die aktiewe laag 'n rol speel in die vatbaarheid daarvan, ondersteun. Dit is bekend dat die metaboliese aktiwiteit van bakteriese selle nader aan die aanhegtingsoppervlak van die biofilm verlaag is,

weens 'n afname in diffusie van suurstof en nutriente in daardie deel van die biofilm. Dit is moontlik dat hierdie differensiasie, wat lei tot die vatbaarheid van die buitenste laag van die biofilm vir ongunstige omgewingstoestande, 'n oorlewingsmeganisme daarstel wat die onderliggende selle beskerm.

Die resultate wat hier voorgelê word, ondersteun verskeie hipoteses wat die verhoogde weerstandbiedendheid van biofilms teen antimikrobiese middels beskryf. Toekomstige werk sluit ondersoeke in na veranderende patrone van geenuitdrukking wat plaasvind wanneer 'n bakterie in aanraking kom met 'n oppervlak, vasheg en ook weer vrygestel word, asook die invloed hiervan op die vermoë om antimikrobiese behandeling te oorleef.

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CONTENTS

Chapter 1

GENERAL INTRODUCTION and OBJECTIVES

The accumulation of microorganisms at natural and artificial surfaces in aqueous environments is a universal and notorious problem. Indeed, it has been said that the survival and reproduction of bacteria in most fluid systems is dependent on the colonization of a surface and integration into a community (Lappin-Scott and Costerton, 1989; Lawrence *et al.*, 1995).

Most solid surface environments with sufficient moisture and available nutrients can provide a suitable niche for biofilm development, and it is estimated that the overwhelming majority of bacteria in these environments are assembled together as functional biofilm communities, rather than living in a suspended form in the bulk fluid (Costerton *et al.*, 1995). Examples of surfaces where biofilms can be found include teeth, medical prosthetic devices such as cardiac pacemakers, the inside of water distribution pipes and on rocks in rivers or streams (Wimpenny, 2000).

Surface attachment allows bacteria to remain in an advantageous niche and prevent dispersal by flowing fluid. As mentioned previously, biofilms are prevalent in natural and man-made environments and for this reason Watnick and Kolter (2000) postulated, as an extreme example, that bacteria in the planktonic phase may be seen as a migrating mechanism, whereby the bacteria leave one surface area only to attach to another.

Having established the ubiquitous nature of biofilms, it is important to place their contribution, whether positive, or negative, into context.

The resilient nature of biofilms is often exploited for the treatment of water containing high concentrations of organic materials. Sanitary engineers encourage the development of biofilms in anaerobic digesters, trickling filters and rotating biological contactors where the metabolic activity of the bacteria can decrease the amount of organic carbon in the water. The attached biomass usually accumulates and detaches until a stable, degradative community is established, which is remarkably resilient to fluctuations in the environment (Costerton *et al.*, 1995).

Biofilm formation can, however, also have a negative impact in the industrial and medical fields. An interesting counter-balance is created as the bacteria are seen to benefit from the

association with the surface, while the surface material is often damaged along with a decline in water quality and reduced efficiency of the surface. Biofilms often clog filters and injection faces, foul products, produce harmful metabolites (e.g. H_2S) and colonize the water-cooled surfaces of heat exchangers, which can reduce the efficiency of heat exchange to less than 10% of the specification values (Costerton *et al.*, 1987). This situation is often referred to as microbial fouling or biofouling (Lappin-Scott and Costerton, 1989).

Industrial companies spend large amounts on biocides and detergents in an attempt to inhibit or remove attached bacterial growth in industrial water systems. These treatment regimens are often unsuccessful due to the refractory nature of biofilms. Coupled to this, is the fact that the planktonic bacteria have in the past received far more attention than the attached forms. The efficiency of antimicrobial treatment has often been measured by sampling the planktonic bacterial numbers before and after treatment, while completely disregarding the contribution of the biofilm (Ludensky, 1998). This situation is rapidly changing as scientists, engineers and medical personnel are becoming aware that attached bacteria are not only more prevalent but also more resistant to antimicrobial treatment.

Several human infectious diseases have been linked to the biofilm mode of growth. Some examples include native valve endocarditis, inflammation of the middle ear (otitis media), cystic fibrosis and periodontal diseases (Donlan and Costerton, 2002). Biofilms can also develop on the surface of medical prosthetic devices, where they can cause severe and persistent infections when antibiotic resistance is coupled with efficient evasion of host immune defenses. Treating the patients with antibiotics might suppress the symptoms of infection by killing the planktonic bacteria, which are released from the attached biofilm, without eradicating the biofilm itself. As soon as the antibiotic treatment is stopped, the biofilm can serve as a source of re-infection (Stewart and Costerton, 2001).

The biofilm may be composed of single or multiple species, depending on the device and its duration of use within the patient. The medical devices differ widely in terms of design and use, but specific factors, such as duration of use, flow rate, and the number and types of organisms exposed to, can influence susceptibility to biofilm formation (Donlan, 2001).

Approximately 90 000 large joint replacements are carried out annually in the United Kingdom. Hospital costs for hip replacements are estimated between £3000 and £4000 each. Hip replacement infections occur after about 1.5-5% of operations, increasing the cost to between £20 000 and £30 000 per patient, and to £100 million per year over-all (Bayston, 2000).

Biofilm related infections pose a serious health risk, and unnecessary expenditure in industrial and medical settings. The resistance of biofilms to antimicrobial treatment further complicates the treatment of these infections.

Controlling biofilm formation is, and will remain to be, a challenge to industry. This task is compounded by the inherent heterogeneous nature if biofilms, as well as their recalcitrance to antimicrobial treatment.

The specific objectives of this study were:

1) To test the hypothesis that attached bacterial populations are more resistant to killing by antimicrobial agents than their associated planktonic counterparts.
2) To isolate and characterize a bacterial strain, capable of forming a biofilm, from a natural environment with a history of exposure to antimicrobials.
3) To compare the effect of growth rate on the antimicrobial susceptibility of the isolated organism, when cultivated as a free-floating population, attached to a surface as a biofilm and when released from the biofilm.
4) To compare the specific average growth rate in a biofilm to that observed in batch culture.
5) To evaluate the efficiency of a fluorescent viability stain that is frequently applied in biofilm-related investigations.

Chapter 2
LITERATURE REVIEW

2.1. The biofilm mode of growth.

2.1.1 The ecology of microbial biofilms.

Microbes are key elements of all ecosystems on earth, and are involved in nutrient cycles as well as decomposition processes. Some authors, such as Carl Woese, have even gone so far as to claim that the microbes in water, soil and those associated with plants, comprise the biggest portion of living biomass on earth and as such, should be regarded as the dominant life-form on earth (Prescott *et al.*, 2002).

The ability of microorganisms to survive throughout the biosphere can be attributed to their extraordinary metabolic capability and phenotypic flexibility. Their survival is also dependent on their ability to select an environmental niche and colonize it, be it by flagellar driven motility, aggregation or attachment.

The ubiquitous nature of biofilms demonstrates the ability of different microbial species to interact with each other in order to survive and propagate in a new micro-niche. Attachment has the added advantage of keeping cells into close contact, thereby facilitating communal interactions so that neighbouring cells can benefit from each other's phenotypic capabilities. Since this is such a universal occurrence, this might be the key to the success of biofilm-forming microorganisms (Davey and O'Toole, 2000).

Biofilms can be composed of single or multiple microbial species, implying that this form of existence is truly dependent on intra- and/or intercellular interactions and communication. The development of the biofilm therefore requires a concerted effort by all of the members in the community, which is reminiscent of the multicellular behaviour observed in eukaryotes. By observing bacteria within the biofilm as part of a community, rather than a single entity, truly novel insights into microbial biology and ecology might be gained (Davey and O'Toole, 2000).

2.1.2 Biofilm development.

The development of a biofilm at a surface involves multiple steps. These steps have been characterized by microscopic observation, using tools such as confocal scanning laser microscopy (CSLM), as well as molecular techniques to elucidate differential gene expression and phenotypic alteration upon attachment.

The traditional approach to the formation of a biofilm postulates that a surface can induce changes in bacterial gene expression, which results in an altered phenotype (Costerton *et al.*, 1995). Once the bacteria are in close contact with the surface, they become immobilised. This immobilisation may take on different forms, such as a monolayer of scattered cells, or clusters of microcolonies interspersed in extracellular polymeric substances (EPS). Some authors believe that the idealized biofilm structure is three-dimensional with mushroom-shaped microcolonies interspersed with fluid-filled channels or pores.

The formation of a biofilm on a solid surface has historically been divided into distinct steps, as summarized by Busscher and Van der Mei (2000). The first step is the formation of a conditioning film, which involves the adsorption of macromolecules to the surface, with evidence suggesting that the conformation and composition of the adsorbed macromolecules depend on the properties of the surface. The second step involves the transport of microbes towards the surface, before initial adhesion occurs, by non-specific van der Waals forces. Initial adhesion is believed to be reversible, but at some point, the attachment becomes irreversible. Attached microbes produce extracellular polymeric substances and divide in order to colonize the surface. A lack of internal cohesive forces or unfavourable environmental conditions may result in the detachment of parts of the biofilm.

The use of microelectrodes to provide information on the physical conditions within regions of the biofilm has contributed towards a better understanding of the life of bacteria at a surface (DeBeer *et al.*, 1994; Walters *et al.*, 2003). Dissolved oxygen concentrations and pH measurements are just some of the physical parameters that can be measured using this technique. The heterogeneous structure of the biofilm leads to an equally diverse distribution of, for example, dissolved oxygen concentrations. The existence of channels and pores within aerobic biofilms allow for the presence of oxygen concentrations, similar to that observed at the biofilm surface, in the deeper layers of the biofilm. The uneven, irregular surface of biofilms was shown to increase the diffusion of oxygen into the biofilm from the bulk liquid (DeBeer *et al.*, 1994). This provides support for the hypothesis that the biofilm structure is not an erratic assembly of cells and EPS, but highly organized to provide for optimal influx of oxygen and nutrients.

2.1.3 The biofilm matrix - Extracellular Polymeric Substances (EPS).

Extracellular Polymeric Substances (EPS) are produced by numerous microorganisms and are frequently associated with the adhesion of microbial cells to a solid substrate and cohesion between individual cells and particles (Sutherland, 1982; Characklis and Wilderer, 1989). It should be noted though, that the biofilm matrix is believed to represent only one of various factors contributing to the accumulation of bacteria at a surface (Sutherland, 1999).

EPS are organic macromolecules, and comprise mainly polysaccharides, and to a lesser degree nucleic acids, proteins, phospholipids and humic material. Microbial polysaccharides can be composed of a virtually unlimited range of combinations of these molecules (Wingender *et al.*, 1999). The EPS found in biofilms vary highly with respect to physical properties and composition, but it remains to be determined whether any of these substances are produced only in a sessile community or during planktonic growth as well (Sutherland, 1999). The surrounding physical environment, including the concentrations of available nutrients, the resident microbes and their level of metabolic activity, determine the structure of the matrix (Sutherland, 2001).

The EPS matrix consists of a large amount of water, even though some exopolysaccharides frequently found in biofilms are probably water insoluble (Flemming and Wingender, 2001). The polysaccharides usually consist of a backbone formed by the polymerisation of similar or identical monomers, which in turn, may be arranged in repeating units with mostly either 1-3 or 1-4 linkages in the α- or the β-configuration. The secondary structure of the polymers are determined by firstly the composition, and secondly the linkages within the backbone of the molecule. The secondary structure frequently takes on a helical formation, and a complex three-dimensional matrix is created when these helices associate with each other. Some polymer networks do not seem to have an orderly composition (Sutherland, 1999). These polysaccharides are regarded as extremely effective in helping to maintain the structural integrity of the biofilm.

The biofilm matrix can be compared to an immobilized enzyme system, as the environment and enzymatic activities within the matrix change regularly in an effort to reach a stable state. This steady state can be altered by intrinsic factors, such as the genetic abilities of the resident microbes, as well as extrinsic factors exemplified by the physico-chemical environment involving diffusion gradients and transport (Sutherland, 2001).

2.1.3.1 Sources of EPS.

The observed diversity in EPS composition, as well as the distribution within biofilms is the result of various processes. While EPS are actively produced by microbial metabolic pathways and secreted, it can also be adsorbed from the surrounding environment. The shedding of cell surface material, or the death and lysis of cells can contribute towards the amount of organic polymers in the environment. These substances can then be incorporated into the EPS of the biofilm, resulting in a 'recycling yard' for metabolic products. The idea of recycling may be taken further, as existing EPS components may be degraded by extracellular enzymes, to provide the bacteria with a carbon or energy source during starvation conditions, or to release the bacteria from the matrix. All of these proposed processes can contribute to the observed diversity in EPS associated with even a single species biofilm (Wingender *et al.* 1999).

2.1.3.2 Substrates for EPS synthesis.

Microorganisms are capable of utilizing a large number of substrates to produce EPS. No relationship has yet been found between the (in) ability of the organism to convert a specific substrate into a polymer with a particular composition.

In strains of *Escherichia coli* and *Enterobacter aerogenes*, grown under limiting nitrogen, phosphorus, potassium or sodium conditions, EPS production was found to be increased, while the composition thereof was independent of the carbon source as well as the nature of nutrient limitation (Sutherland, 1982). In general, it could be postulated that higher concentrations of easily metabolised nutrients lead to a biofilm of much greater density than those produced in oligotrophic environments (Sutherland, 2001).

2.1.3.3 The importance of multivalent cations.

Various ions, such as magnesium, calcium and potassium, are known to be essential for either the uptake of substrates for EPS synthesis, or are required as cofactors in the synthesis process itself (Sutherland, 1982). Calcium, copper or iron have also been found to act as important bridging ions within the EPS structure, thereby increasing its stability and ordered conformation by the formation of ionic bridges (Sutherland, 1999; Flemming and Wingender, 2001). The polymers could also serve a useful role in trapping and maintaining a reservoir of ions to supplement deficiencies during oligotrophic growth conditions (Sutherland, 1999).

2.1.3.4 Functions of EPS.

2.1.3.4.1 Structural and functional integrity.

Extracellular polymeric substances are mainly responsible for the structural and functional integrity of the biofilm and are considered as the key components that determine the physicochemical and biological properties of biofilms (Flemming and Wingender, 2001).

2.1.3.4.2 Protection from desiccation.

The EPS matrix is highly hydrated, especially in the vicinity of the microbial cells, and could protect the cells from desiccation during dry periods (Sutherland, 1999).

2.1.3.4.3 Genetic transfer.

Wolfaardt *et al.* (1999) argued that there is some, though mostly indirect, evidence that EPS plays a role in the transfer of genetic material. The EPS matrix brings the bacterial cells into close contact with each other and alters the hydrodynamic conditions, which may facilitate the transfer of genetic elements between bacteria. It is now known that the transfer of genetic material between bacteria is enhanced in many biofilms and the process has been linked to the developmental process of the biofilm itself (Molin and Tolker-Nielsen, 2003).

2.1.3.4.4 The trapping of nutrients within the EPS matrix.

Several researchers have suggested that the EPS matrix may serve as a repository of nutrients. The EPS may be involved in the trapping of nutrients from the oligotrophic environment, being especially relevant to biofilm communities, since the bulk flow of fluid, containing nutrients, is not directly accessible to the bacterial cells and their extracellular enzymes. Once the nutrients have accumulated within the matrix, it can then be degraded by secreted enzymes and the products taken up by the microbial cells (Sutherland, 1999). This could be an adaptive strategy used by bacteria when living in low-nutrient environments, to enhance survival. Bengtsson (1991) reported that ground-water bacteria reduced the production of EPS by more than half in response to the addition of glucose and phosphate to pure ground water. When exposed to oligotrophic conditions, bacteria attached to soil particles produced approximately 6 times more EPS than the free-floating population of cells, although the composition of the EPS was similar for both.

Wolfaardt *et al.* (1995) provided evidence for the accumulation of the herbicide diclofop methyl and its aromatic breakdown products in the EPS of a degradative microbial consortium, when it was provided as the sole carbon source. When a labile carbon source was provided, the trapping of the herbicide was reduced 2.2 fold. The accumulation of diclofop methyl was shown to be the result, at least in part, of metabolic activity and not only a consequence of a passive diffusion process or binding to EPS components. When starvation conditions were induced by the removal of the carbon source, the diclofop accumulated in the EPS was degraded by the microbial community.

In a related publication, the same authors reported on the difference in composition and spatial arrangement of exopolymers produced by the same degradative microbial community, when cultivated in the presence of diclofop methyl and a more labile carbon source (Wolfaardt *et al.*, 1998). Nine different fluorescently labelled lectin probes were used to identify EPS components and to determine their position within the biofilms. Biofilms grown on the labile Tryptone Soy Broth (TSB) carbon source showed a more even distribution of lectin binding sites, while the glycoconjugates of the diclofop methyl-grown biofilm were more heterogeneously dispersed in the vertical and horizontal planes. The difference in EPS composition and structure between the diclofop methyl and TSB-grown biofilms indicated that the EPS play an important part in the structure of a biofilm community.

2.1.3.4.5 Protection from antimicrobial agents.

The protective barrier provided by the EPS may be responsible for the ability of biofilms to withstand antimicrobial treatment. The application of sub-lethal concentrations of specific antimicrobials lead to the increased production of EPS even though bacterial growth is inhibited. This is of particular relevance in industrial systems where routine dosages of ineffective concentrations of antimicrobials could allow microbial surface colonization, as well as enhanced EPS production to continue; ultimately leading to increased resistance to subsequent treatment cycles (Sutherland, 1999).

In conclusion, the existing information of the role played by EPS within biofilm communities, is rather limited. Sutherland (1999) suggested that each multispecies bacterial biofilm should be regarded as unique, as it exists and creates its own microenvironment. The vast difference in composition of exopolysaccharides, produced by individual organisms, or several different organisms, provides numerous opportunities for investigation.

The biofilm matrix unites all the essential components of the biofilm – the microbial cells, water, polysaccharides, nutrients and waste products. The cells are heterogeneously dispersed within the EPS matrix, while the rest is subject to constantly changing diffusion patterns, resulting in various gradients and microenvironments. This is an inherent characteristic of the natural biofilm, and no doubt contributes towards its multicellular functionality.

2.2. Comparing the planktonic and biofilm phenotypes.

In a minireview published in 1994, Costerton and co-authors stated: "It is, clearly, timely to ask the question 'What are the essential differences between a planktonic cell growing in the conventional batch culture and a cell of the same species growing in a natural multispecies biofilm?' " (Costerton *et al.*, 1994).

The authors continued by pointing out several important observations. The first was that sessile communities generally consisted of more cells than that of the free-floating population in the bulk-liquid phase (Geesey *et al.*, 1977). The use of confocal scanning laser microscopy (CSLM) allows the visualization of successive focal planes in living biofilms, without dehydration or fixation, as is the case with electron microscopy. CSLM examinations revealed the presence of microcolonies, where the bacteria are densely packed within EPS, interspersed with less dense regions and water-filled channels (Lawrence *et al.*, 1991; Caldwell *et al.*, 1992). These channels have even been likened to a primitive circulatory system, similar to that of multicellular organisms (Costerton *et al.*, 1994).

The microenvironment is a fundamental characteristic of biofilms, since biofilm bacteria live in diffusion-limited environments. Different microenvironments exist across the biofilm, leading to various gradients of nutrients, oxygen and metabolic products. These gradients can, in turn, influence metabolic activity, microbial diversity and the distribution of cells throughout the biofilm matrix. The microenvironment itself can therefore be held responsible for much of the naturally observed diversity of microbes (Lawrence *et al.*, 1996). This is in contrast to what suspended cells would experience when continuously agitated in fluids.

The diffusion process within biofilms was described by Lawrence *et al.* (1994) by means of CSLM and size-fractioned fluorescent dextrans. The diffusion rate of the dextrans through the biofilm was shown to be less than that observed within the bulk liquid, indicating that the diffusion process was restricted by components of the mixed-species biofilm. Differential rates of diffusion in different parts of the biofilm provided an indication of the spatially heterogeneous character of the biofilm structure. To illustrate this point, the presence of

actively respiring *Klebsiella pneumonia* and *Pseudomonas aeruginosa* cells within biofilms were identified using the fluorogenic redox indicator 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) together with 4', 6-diamidino-2-phenylindole (DAPI). Spatial gradients of respiratory activity were identified within intact biofilms upon disinfection treatment with monochloramine (Huang *et al.*, 1995).

The immense structural complexities of natural biofilms develop as a result of several factors both innate and external to the biofilm. External factors, such as the properties of the attachment surface, nutrient availability, flow rates, grazing and environmental stresses can influence the structural features of the biofilm. The internal determining factors include microbial behavior, metabolic capability, the ability to sense and respond to environmental stimuli and interactions between community members (Lawrence *et al.*, 1996). As a consequence of their transient positions in fluid systems, planktonic microbes are unable to benefit from lasting interactions with neighboring cells.

The metabolic cooperation between individual cells within a multispecies biofilm is made possible by the anchored nature of the biofilm, as well as the unique microenvironment created by limited diffusion of nutrients and metabolic products. The organized nature of the biofilm structure and metabolic collaboration almost resemble what is seen in primitive multicellular eukaryotes and could account for the extraordinary metabolic capabilities observed in biofilms (Costerton *et al.*, 1994).

The metabolic activity of the cells in the biofilm could theoretically be controlled by the environmental conditions at the surface, or the expression of specific genes induced by adhesion, or by growth as a biofilm. Current investigations into the genetic basis of biofilm formation and development have identified several genes that are involved in the formation of a biofilm and have been reviewed recently by Pratt and Kolter (1999).

Of the Gram-negative bacteria studied to date, namely *E. coli, Pseudomonas fluorescens, P. aeruginosa* and *Vibrio cholerae*, genes encoding for flagella have been found to be involved in the formation of biofilms under specific environmental conditions (O'Toole and Kolter, 1998a; Pratt and Kolter, 1998).

Twitching motility involves the translocation of cells across a surface and is mediated by surface appendages termed type IV pili. The pili have been implicated in the biofilm formation process of *P. aeruginosa* (O'Toole and Kolter, 1998a). Both these observations have led to the hypothesis that flagellar driven motility allows the bacteria to reach and

contact the surface, whereas twitching motility is involved in the early structural development of the biofilm.

The presence of flagella and pili are, however, not always essential to the ability to attach. In rich culture media, such as Luria Bertani broth, biofilm formation by *V. cholerae* is enhanced by motility, but motility-negative mutants are capable of forming biofilms even at a rate slower than that of the wild type (Watnick and Kolter, 1999). In support of this, the inability of *P. fluorescens* to form biofilms when flagellar motility is interrupted can be reversed by cultivation in the presence of citrate, glutamate or high iron concentrations (O'Toole and Kolter, 1998b).

In addition to the discovery of the involvement of flagella and pili in biofilm formation, a role for the surface adhesin, Curli, in the attachment of a non-motile *E. coli* strain was identified (Vidal *et al.*, 1998). This observation led to an addition to the above-mentioned hypothesis, in that motility facilitates the movement of bacteria towards the surface as well as initial interaction, whereafter the production of adhesins allow for stability and biofilm formation. Another structure identified as being involved in biofilm formation includes the mannose-sensitive type I pilus of *E. coli* (Pratt and Kolter, 1998; Watnick *et al.*, 1999).

Others have proposed a role for the quorum-sensing signal molecules, acylated homoserine lactones (acyl-HSLs) in the development of biofilms (Davies *et al.*, 1998; Vuong *et al.*, 2000; Li *et al.*, 2002). Davies *et al.* (1993) showed that an extracellular signal molecule, N-3-oxododecanoyl-L-homoserine lactone ($3OC_{12}$-HSL) encoded for by the *lasI* gene, is involved in the formation of biofilm structure. A *lasI* mutant strain of *P. aeruginosa* formed a densely packed biofilm, 80% thinner than the wild-type biofilm and without microcolony and water channel formation. The authors speculated that attached bacteria could only differentiate to form a three-dimensional biofilm once a critical amount of biomass was present at the surface to produce a threshold concentration of the quorum-sensing signal molecule.

Several other genes and metabolic pathways have been preliminarily identified as being involved in bacterial biofilm formation. The few examples mentioned above and several others are reviewed by Pratt and Kolter (1999). As a summary of what is known to date about the involvement of specific genetic pathways in biofilm formation and development, they concluded that since the biofilm mode of growth is so ubiquitous, it could be equated to a complex multicellular organism. This is in contrast to the apparent unicellular existence typical of planktonic bacteria and this realization has perhaps been the most important step forward in the quest to understanding biofilm formation at the genetic level.

Bacterial aggregates, as typified by sludge granules in anaerobic digesters used to treat sewage and industrial wastewaters, are an example of the advantages obtained through adhesion, whether it occurs between members of different microbial species or to solid surfaces. Adhesion provides a stable association and allows for the creation of suitable environmental conditions necessary for certain metabolic reactions to occur. MacLeod *et al.* (1990) observed a three-layered structure within reactor granules, each with a distinct assemblage of different bacterial species and associated extracellular polymers. The spatial and metabolic co-operation between the members of the consortium allowed for the conversion of a complex substrate into carbon dioxide in the center of the granules. The cooperation between the species created an oxygen gradient, such that completely anaerobic organisms could grow in the center of the granule. This example of a mutually beneficial cooperation between microbes with different metabolic capabilities aptly illustrates the benefits provided by adhesion, which would not be available to planktonic microorganisms.

2.3. Antimicrobial resistance.

2.3.1 The history of antibiotics.

Louis Pasteur established the *germ theory of disease* in the second half of the 19th century and Robert Koch identified and cultured the first disease causing bacteria. Paul Ehrlich was the first to put forward a theory of selective toxicity in 1910. He envisioned therapeutic agents, which would damage only the infecting microorganism but not the healthy cells of the patient. He called these agents 'magic bullets' (Van den Ende, 1987). In the next decade, several of these 'magic bullets' were discovered.

In 1935, Professor Gerhard Domagk discovered the antibacterial action of a sulphanilamide, used as a leather dye named Prontosil Red (4-sulphonamide-2', 4'-diaminoazobenzene hydrochloride). The discovery of Prontosil opened up undreamed-of prospects for the treatment of infectious diseases (Nobel Lectures in Physiology or Medicine 1922-1941).

Treatment of streptococcal, pneumococcal and gonococcal infections with sulphonamides was effective at first, until the 1940's when failures in the treatment of gonorrhea were reported. Soon thereafter resistant streptococci and staphylococci were also observed.

The first true antibiotic (i.e. the natural product of a microorganism) discovered, was penicillin. It was discovered initially by Professor Alexander Flemming in 1928 and then

rediscovered by Professor Howard Florey and Doctor Ernst Boris Chain in 1938. The first patient to be treated with penicillin in 1941 showed a good initial response, but died because insufficient amounts of the drug were available. Bacteria insensitive to penicillin were observed as early as 1940. Some bacteria were even able to deactivate crude antibiotic preparations. The introduction of penicillin in animal feeds during the 1950's worsened the situation. In the early 1960's, only about 10% of staphylococci strains were resistant to penicillin G, but today almost all the strains are resistant to most of the simpler penicillins (Mann and Crabbe, 1996).

Dr. Selman Waksman isolated the first aminoglycosidic antibiotic, streptomycin, from the soil microbe *Streptomyces griseus* in 1943. The streptomycin patent was recently named one of 10 patents that shaped the world. Streptomycin showed activity against *Mycobacterium tuberculosis*, the causative agent of tuberculosis, and as such yielded great hopes for the eradication of the disease. Waksman coined the term 'antibiotic' in 1953 and defined it as "a chemical substance, produced by microorganisms, which has the capacity to inhibit the growth and even to destroy bacteria and other microorganisms, in dilute solutions."

The discovery of streptomycin was soon followed by the discovery of other aminoglycoside antibiotics such as tetracycline, chloramphenicol and erythromycin. The first cephalosporin (an antibiotic closely related to penicillin) produced by a mould, was discovered by Brotzu from a sewage outlet in the Sardinian sea. These discoveries led to imposing predictions of eradicating infectious diseases from the human population by eliminating the bacteria causing it.

The emergence of penicillin resistant staphylococci in the early 1950's and its spread soon dashed these ideas of eliminating pathogenic bacteria. The next sobering finding was the isolation of bacteria with resistance against multiple, unrelated antibiotics, termed multiple resistance. These findings resulted in severe clinical limitations, applicable even today, namely a decrease in the number of effective antibiotic agents and restrictions on those agents that can be used, as well as a requirement for the prescription of more toxic and more expensive agents (Van den Ende, 1987).

The era of antibiotics started in 1939 and within 18 years approximately 30 antimicrobial agents were discovered, developed and prescribed. More than 1 220 antimicrobial compounds, in various stages of development and clinical trials, have been reported or reviewed in the American Society for Microbiology Journal *Antimicrobial Agents and Chemotherapy* (AAC) from 1972 to 1998 (TABLE 2.1).

TABLE 2.1: Categories and percentages of Antimicrobial agents reported in AAC between 1972 and 1998 (Swartz, 2000).

	Antimicrobial Agents	
Category	**Number**	**Percentage**
Antibacterial	567	46%
Antiviral	293	24%
Antiparasitic	158	13%
Antifungal	110	9%
Antimycobacterial	92	8%
Total	1220	100%

The proportion of newly developed antibacterial drugs as a percentage of all antimicrobials produced, that has been reported in AAC, has declined from 62% in 1972 to 26% in 1998 while the relative abundance of new antiviral drugs has increased dramatically between 1997 and 1998 alone, to account for 31% of all antimicrobials produced during that year. While the numbers of antimicrobial drugs reported in the AAC have increased, a definite need for clinically approved new drugs that are effective against antimicrobial-resistant pathogens exists (Swartz, 2000).

Resistance is defined as the ability of a microorganism to grow in the presence of an elevated level of an antimicrobial (Lewis, 2001). Several antimicrobial targets in bacteria are altered by changes in the immediate environment of the bacterial cell, thereby reducing the potency of the antimicrobial.

Antibiotics and biocides are all classified as antimicrobials, but a distinction between these two classes is made according to the specificity of action and degree of toxicity to eukaryotic tissue. Antibiotics usually have a specific cellular target that is not shared with the infected host, while biocides act against any number of targets, each with different susceptibilities and which may be present within mammalian systems. While the elevated resistance of microorganisms towards various antibiotics has been increasingly reported, little is known about the response of microbes towards the use of biocides during the last 100 years.

Small changes in the minimum inhibitory concentration (MIC) of biocides towards microbes can be achieved by deploying sublethal concentrations of these agents. The increase in the MIC generally does not affect the outcome of the treatment, as it does with antibiotics, since

most biocides act at multiple target sites, and these agents are applied at concentrations far exceeding that of the MIC (Gilbert *et al.*, 2002a). The immense increase in the use of household products containing biocides has lead to fears that this might expand the prevalence of antibiotic resistance in the environment, in instances where the antibiotic and biocide have a shared target site.

While the ability of individual cells to resist killing by antibiotics have been investigated, and several contributing factors identified, an increasing amount of attention is being given to the ability of microbial biofilms to resist antimicrobial treatment. It has been reported extensively that organisms in the biofilm mode of growth are more resistant to antimicrobial treatment than free-floating organisms. This reported phenomenon is widely regarded as the rule rather than the exception and much has been said about the role that sessile microbes play in determining resistance to antimicrobials in device-related infections, as well as the fouling of industrial water systems (Vrany *et al.*, 1997; Stewart *et al.*, 1998; Broon *et al.*, 2000; Walters *et al.*, 2003).

When antibiotics are prescribed to a patient, an initial high concentration-loading dose is administered followed by subsequent maintenance dosages of lower concentrations, at regular intervals. Anwar *et al.* (1992) examined the effect of such a treatment regimen on young and old biofilms of *Staphylococcus aureus* cultivated within a chemostat system, containing floating attachment surfaces. The antibiotics, tobramycin and cephalexin, were incorporated into the culture media of young (2 days) and old (21 days) biofilms for the initial loading dose. Lower concentrations of antibiotics were added at 6-hour intervals for 7 days, to simulate maintenance dosages. On the seventh day after the loading dose was first applied, the antibiotic containing culture media was replaced with antibiotic-free media to investigate regrowth of the biofilm. Planktonic and young biofilm cells were highly susceptible to the initial dose of antibiotics, with only 0.0002 % viable planktonic and 0.005 % viable biofilm cells surviving treatment, as determined with standard plate counts. No viable cells could be detected either in the chemostat fluid or on the attachment surfaces after the second maintenance dose of antibiotics.

The viability of planktonic cells of the 21-day old chemostat system was reduced to 0.0005 % after antibiotic treatment, while sessile *Staph. aureus* was reduced to 2.5 % viable cells. The addition of two maintenance dosages decreased the planktonic population to approximately 10 viable cells per millilitre, while four maintenance dosages resulted in 0.002 % viable biofilm bacteria. The level of viable biofilm bacteria did not decrease with further application of maintenance dosages over the period of six days. When fresh culture media was

introduced, the number of viable planktonic cells increased to pre-treatment levels within 3 days, while the biofilm population recovered to 2 % of the original total amount of viable cells within the same period (Anwar *et al.*, 1992).

This study illustrates the difficulty in eradicating adherent populations of bacteria. While the initial and maintenance dosages of antibiotics are sufficient to kill all planktonic cells, it does not destroy all of the viable biofilm bacteria. The decrease in planktonic numbers results in an apparent alleviation of the clinical symptoms of the disease, leading the physicians to believe that the antibiotic application was successful in eradicating the pathogen. Once the treatment is halted, surviving biofilm bacteria are able to revive, grow and shed new daughter cells, which in turn could lead to a recurrence in the symptoms of infection.

2.3.2 Cellular mechanisms of resistance.

2.3.2.1 Acquisition of foreign genetic material.

The transfer of genetic material between phylogenetically diverse bacterial species has been shown to occur and is known as horizontal gene transfer. Genes encoding for resistance to antimicrobials have been found on plasmids, transposons and integrons. Once one of these mobile genetic elements has entered the cell, it could potentially integrate into the bacterial chromosome and be inherited by subsequent daughter cells (White and McDermott, 2001; Hogan and Kolter, 2002).

In a recent publication, Molin and Tolker-Nielsen (2003) concluded that the recurrent and competent transfer of genetic material by transformation and plasmid conjugation occurs in many bacterial biofilms. Gene transfer has been shown to be enhanced in biofilms, as well as being linked to the development of biofilms. This gives biofilm bacteria the advantage of being able to adapt to environmental stresses, such as the presence of antimicrobials and other toxins, through horizontal gene transfer.

2.3.2.2 Deletion or modification of the target site.

The evolutionary process has allowed bacteria to respond to the increased worldwide use of antibiotics. The presence of an antimicrobial agent provides a selective advantage to those individuals in the population, which can counter the action of the antimicrobial. A mutation in the antimicrobial target site might render the agent ineffective. An example of this mechanism is found in some Gram-positive bacteria with altered peptidoglycan structures, which negates the bacteriostatic action of glycopeptide antibiotics (Wright, 2003). In

addition, the presence of the antibiotic may lead to the overexpression of the target site. As a result, the effective concentration of the agent would be decreased, allowing for the survival of some members of the population.

2.3.2.3 Enzymatic inactivation of the agent.

The most frequently encountered mechanism of resistance against β-lactams is the enzymatic hydrolysis of these antibiotics by β-lactamases leading to a loss of function. Although native β-lactamases are present on the chromosomes of many bacteria, resistance towards β-lactam antibiotics can also be gained through the acquisition of plasmids or transposons with the β-lactamase genes, or by derepression or overexpression of chromosomal genes by mutation or natural processes (Livermore, 1995).

2.3.2.4 Multidrug efflux pumps.

Several classes of efflux pumps have been identified which provide protection from heavy metals and antimicrobial agents by actively exporting these compounds from the cell before harm can be inflicted.

The presence of a stress-induced efflux-system has been identified in *E. coli*. The *acrA* and *acrB* genes encode for a membrane fusion protein and an inner membrane efflux transporter, respectively, and mutations in either result in the increased susceptibility of *E. coli* to a range of compounds. Expression of *acrA* and a*crB* is induced during periods of stress, such as stationary growth, elevated levels of ethanol and NaCl. Transcription also appears to be regulated by *marR*, a component of the multiple antibiotic resistance regulon (*mar* regulon) (Ma *et al.*, 1995).

The efflux-systems require energy in the form of either ATP or a proton motive force to export harmful compounds out of the bacterial cell. These systems can be located on the native bacterial chromosome, or acquired from another bacterium by horizontal gene transfer. The AcrAB pump mentioned above has been implicated in multiple antibiotic and biocide resistance mechanisms, against such compounds as quaternary ammonium compounds, triclosan and chlorhexidine (Levy, 2002).

2.3.3. Mechanisms of biofilm resistance to antimicrobials.

Several extensive review articles (Lewis, 2001; Mah and O'Toole, 2001; Stewart and Costerton, 2001; Gilbert *et al.*, 2002a; Gilbert *et al.*, 2002b) have been published recently, which summarise the various proposed mechanisms whereby biofilm organisms are able to resist antimicrobial treatment when their planktonic counterparts cannot.

These proposed mechanisms often cannot individually account for the observed antimicrobial resistance of biofilms, since the mechanisms of action are often interdependent, as well as firmly linked to the inherent multicellular characteristics of biofilms.

2.3.3.1 EPS.

The ability of biofilms to survive in the presence of a range of unfavourable conditions and antimicrobial agents has repeatedly been attributed to the EPS matrix enclosing the microbial cells (Costerton *et al.*, 1995). It has been shown that EPS biosynthesis is induced when bacteria attach to a surface, an example of which is alginate production in attached *P. aeruginosa* (Davies *et al.*, 1993).

The production of EPS by slow growing, attached *Staphylococcus epidermidis* was greater than that produced by planktonic cells, even though cultivation took place under carbon-limited conditions. From these observations it was concluded that surface attachment not only induces EPS production, but that production is inversely related to growth rate. The bacteria released from the biofilm upon division did not remove any of the EPS from the biofilm, but instead it remained associated with the biofilm (Evans *et al.*, 1994). The presence of slow-growing bacteria in the deep recesses of the biofilm could produce more extensive EPS, thereby providing even greater protection.

Several possible mechanisms whereby EPS is involved in providing protection against antimicrobial agents have been identified:

2.3.3.1.1 Diffusion limitation in the biofilm.

In a recent commentary, Stewart (2003) shed light on the fundamental role played by diffusion within a biofilm. Diffusion in biofilms is usually limited, since the flow of fluids is reduced by the dense EPS matrix containing the microbial cells, as well as the distance that solutes need to cross in order to reach the surface. In contrast, planktonic cells are generally agitated by the fluid, which provides the necessary action to transport solutes to the cell.

Key processes within biofilms can be explained in terms of the diffusion process. Examples include cometabolism between metabolically diverse microbes, differential patterns of gene expression, resistance to antimicrobial agents, biocorrosion by microbes and changes in the physiological state of the resident microbes. Delayed or incomplete penetration of antimicrobial agents can also occur as a result of interaction with or adsorption to the biomass, coupled to slow diffusion (Stewart, 2003).

The antimicrobial activity and penetration of two fluoroquinolone antibiotics, levofloxacin and ciprofloxacin, into *P. aeruginosa* biofilms were determined by Vrany *et al.* (1997). The transport of both antibiotics to the base of the biofilm was rapid as determined by attenuated total reflection Fourier transform infrared spectroscopy (ATR/FR-IR). This did not, however, increase the killing efficiency of either of the antibiotics against the biofilm, even though a reduction in cell numbers was observed for planktonic *P. aeruginosa*. This led the authors to speculate that the observed resistance of the biofilms towards the fluoroquinolones could be attributed towards the physiological differences between an attached and planktonic life-style.

Stewart *et al.* (1998) used alginate bead entrapped *E. aerogenes* to test the killing efficacy of four biocides, namely chlorine, glutaraldehyde, an isothiazolone and a quaternary ammonium compound. They found that *E. aerogenes* cultivated in suspension was more susceptible than the alginate entrapped bacteria after treatment with all four biocides. Having established that the alginate bead artificial biofilms display the antimicrobial resistant phenotype of conventional biofilms, the authors investigated whether a proposed mathematical formula, which takes into account an increase in bead radius (analogous to biofilm depth) and cell density, could be used to explain the observed recalcitrance. They firstly hypothesized that biocide transport would be hampered by an increased amount of matrix material, and secondly, that a greater density of bacterial cells would result in the inactivation of the antimicrobial, particularly the highly reactive oxidising biocides.

The results indicated that transport restriction does have an impact on the action of not only oxidising biocides, such as chlorine, but also on non-oxidizing glutaraldehyde, isothiazolone and the quaternary ammonium compound. The mathematical formula did indicate that factors, other than the transport limitation of biocides, played a role in protecting the bacteria (Stewart *et al.*, 1998).

2.3.3.1.2 Adsorption of agents to the EPS matrix.

It has been postulated that the binding of positively charged aminoglycosides to the biofilm EPS matrix can retard the penetration of the antibiotics into the biofilm. By reducing the free concentration of the agent, the driving force behind the diffusion process is negated, possibly providing an explanation for the recalcitrance of sessile populations to antimicrobial treatment. The first demonstration of this concept was provided by the observations of Nichols *et al.* (1988).

These authors demonstrated the binding of the antibiotic tobramycin to alginate and exopolysaccharides from two mucoid *P. aeruginosa* strains. The binding process showed saturation tendencies, which was dependent on the tobramycin concentrations applied. The binding of the agent to the EPS could, however, not be identified as the sole determinant for biofilm resistance.

The fluorogenic redox indicator 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) was used to locate actively respiring cells in intact *P. aeruginosa* and *K. pneumonia* biofilms in response to treatment with 2 mg/l of monochloramine. While respiring cells dominated throughout the biofilm before disinfection, non-respiring cells appeared at the biofilm bulk-liquid interface 30 minutes after monochloramine was added to the growth medium. One hour after treatment was commenced, a significant number of non-respiring cells were also present in the deeper layers of the biofilm and some of the biofilm biomass was lost due to detachment. Only a small fraction of the biofilm contained respiring cells at the end of the two-hour treatment period. The most important observation made was that the loss of respiratory activity was not consistent throughout the biofilm as most of the cells near the biofilm-fluid interface lost activity, while cells near the attachment surface, or within cell clusters, were most active. This observation was attributed to the consumption of the disinfectant within the biofilm due to a reaction-diffusion reaction and/or the presence of essential nutrient gradients resulting in slow-growing cells being more recalcitrant to antimicrobial treatment (Huang *et al.*, 1995).

All of the diffusion-related mechanisms described above contribute to the resistance of biofilm communities towards antimicrobial agents. Most studies have been conducted using single isolates of model organisms, such as *P. aeruginosa*. In most non-medical situations, however, the situation is certainly more complex. Multi-species biofilm formation leads to the production of chemically diverse EPS, with respect to hydrophobicity, composition and charge distribution. This results in a biofilm with extended spatial diversity and physiological gradients of cellular activity. All of these factors contribute towards the complexity of

controlling unwanted biofilm formation, as well as confounding laboratory studies into the mechanisms of the biofilm mode of growth.

2.3.3.1.3 Enzyme mediated reaction-diffusion limitation.

The production of neutralizing enzymes by resistant bacteria could lead to the inactivation of the antimicrobial agents before it can inflict any damage. The biofilm EPS matrix could actually trap the enzymes, leading to a higher concentration around the biomass, and theoretically even greater protection against the antimicrobial.

The induction of hydrolytic β-lactamase production in *P. aeruginosa* biofilms, and its continued association with the biofilm, was shown in the presence of two β-lactam antibiotics, imipenem and piperacillin. Imipenem was the better inducer, although the level of β-lactamase produced within the biofilm was significantly ($P < 0.02$) lower than that synthesized by planktonic cultures of the same bacteria (1 860nU vs. 2 400nU). It was concluded that diffusion of the inducers could have been retarded by the exopolysaccharide matrix or hydrolysed as it passed through the biofilm matrix (Giwercman *et al.*, 1991).

2.3.3.2 Retarded growth within the biofilm.

The existence of a unique biofilm physiology has been suggested to be responsible for the observed resistance to antimicrobials. Compared to the physiology of planktonic cells, the biofilm physiology may involve a slow rate of growth, as a consequence of oxygen and/ or nutrient limitation or high density of cells in the biofilm (Gilbert *et al.*, 1990; Vrany *et al.*, 1997). The layered structure of a biofilm allows for the existence of oxygen, nutrient and waste product gradients, as previously described. Limited concentrations of these essential components can lead to retarded growth within the deeper layers of the biofilm, and in turn to a greater resistance to antimicrobial agents, as the rate of growth has repeatedly been linked to antimicrobial susceptibility.

Slow rates of growth are associated with retarded replication, but not necessarily with low metabolic activity, as reported by Nold and Ward (1996) who observed high rates of metabolic activity in cyanobacterial mats found in hot springs.

The effect of several antibiotics from different classes, were tested against non-growing planktonic *E. coli* cultures and against sessile populations (Ashby, 1994). Some of the

antibiotics tested, such as gentamycin, imipenem (a carbapenem) and all cephamycins tested, except cefotetan, were effective against actively growing as well as non-growing cells. Similar results were observed when these antibiotics were tested against biofilms. This indicated that non-growing cells exhibited susceptibility, similar to that of biofilm bacteria towards the antibiotics tested. However, none of the applied antibiotics were able to reduce the viability of all of the adhering cells, and biofilm regrowth in the absence of the antimicrobial was observed even after treatment with the most effective antibiotics.

In a recent publication, it was shown that cells deprived of oxygen and glucose within *K. pneumonia* biofilms enters a non-growing phase, comparable to stationary growth (Anderl *et al.*, 2003). The application of ampicillin and ofloxacin at 10 times the MIC resulted in the rapid killing of actively growing cells (i.e. exponential phase) within a batch culture, but the cells within the biofilm were not killed by the same treatments, despite complete penetration. The results obtained for the biofilm studies were imitated by testing susceptibility of stationary phase cells against ampicillin when deprived of a carbon and nitrogen source. The combination of nutrient deprivation and near stationary phase existence of some cells within the *K. pneumonia* biofilm could explain their resistance to ampicillin.

The influence of growth rate on antimicrobial susceptibility of biofilms has also been examined for fungal cultures. A perfused biofilm fermenter (Gilbert *et al.*, 1989) was used to cultivate and control the growth rate of *Candida albicans* biofilms and test the effect of various antifungal agents against the yeast. Only one of the agents tested, amphotericin B, resulted in significant decreases in the viable count of biofilm daughter cells when applied at 20 times the MIC. Thereafter, the growth rate of the adherent biofilm was adjusted to determine what effect, if any it had on the ability of the biofilm to resist antifungal treatment. At all three growth rates tested, similar declines in the number of viable daughter cells in the effluent was detected, although at the lowest rate employed ($0.02h^{-1}$) the biofilm continued to shed steady-state numbers of viable cells for 30 minutes before a decline was observed. This could suggest a delayed effect of antimicrobial killing at low rates of growth. In comparison, the resistance of planktonic cultures were shown to be dependent on growth rate, where the cultures exhibited the same decreased susceptibility as biofilms, when growing at extremely slow rates.

Resuspended biofilms were less resistant than intact ones, but more resistant than planktonic cultures when grown at rates higher than $0.2h^{-1}$. The eluted daughter cells were more susceptible than intact and resuspended biofilms at all of the growth rates tested. The resistance of the biofilm to antifungal agents at all growth rates tested indicated that low rates

of growth are not solely responsible for the observed recalcitrance of biofilms towards antimicrobial agents (Baillie and Douglas, 1998).

A more recent hypothesis to account for biofilm resistance to antimicrobials, which relates to slow growth in deeper biofilm layers, involves the induction of the general stress response (GSR). This state is thought to be induced by severe nutrient limitation and results in the reduction of metabolic activity. The presence of even a relatively small portion of the cells within a biofilm expressing this phenotype, might account for the ability to resist antimicrobial treatment (Gilbert *et al.*, 2002a).

2.3.3.3 Multiple antibiotic resistance.

The multiple antibiotic resistance (*mar*) operon is a global regulator that governs multi-drug efflux resistance systems in *E. coli*. It has been proposed that retarded penetration of antimicrobial agents, coupled to slow growth deep within the biofilm, could provide a sufficient amount of time for the bacteria to initiate a 'stress' response, thereby inducing, amongst others, multi-drug resistance systems (Maira-Litran *et al.*, 2000).

As described in Section 2.3.2.4., the expression of the *acrAB* encoded efflux pump in *E. coli* is regulated by a component of the *mar* regulon. The level of expression of the *mar* operon was determined by monitoring β-galactosidase (*lacZ*) activity of a $marO_{II}$-*lacZ* reporter gene fusion construct. The expression of the *mar* regulon was shown to be dependent on the growth phase when grown in batch culture. Chemically defined growth media led to maximal expression in the mid-logarithmic phase, while cultures in nutrient rich media (Luria Bertani Broth) showed increased expression in stationary phase. Expression in continuous chemostat culture was inversely related to specific growth rate, while expression in the biofilm was low and comparable to levels within stationary cultures grown in batch on chemically defined media. This may indicate that oligotrophic environments, resulting in slow growth, could lead to an increase in the expression of the *mar* regulon. This observation could be extended towards slow growing bacteria in the deeper layers of the biofilm with increased resistance to antimicrobials due to increased expression levels. Even so, the authors found that the possible induction of expression within slow growing regions of a biofilm would be insufficient to account for the increased antimicrobial resistance of biofilms (Maira-Litran *et al.*, 2000).

2.3.3.4 The presence of persister cells.

Spoering and Lewis (2001) provided evidence that planktonic *P. aeruginosa* cells in stationary growth phase were more resistant to antibiotic treatment than biofilm cultures, and speculated that this is largely due to the presence of 'persister cells'. The authors oppose the current contention that biofilms can resist higher concentrations of antimicrobials than planktonic cells. They proposed that biofilms are better able to resist killing by antimicrobials and attribute this to tolerance, rather than resistance.

In a related publication, the authors reported that the majority of *P. aeruginosa* biofilm cells were eliminated at low concentrations of fluoroquinolone antibiotics, leaving a small number of cells behind that was not killed by a further increase in antibiotic concentration. They attributed the extraordinary ability of biofilms so survive antimicrobial treatment to the presence of this small fraction of persisting cells (Broon *et al.*, 2000).

In the subsequent study by Spoering and Lewis (2001), the ability of biofilm *P. aeruginosa* cells, as well as planktonic cells in both the logarithmic and stationary growth phase, were compared with respect to their ability to survive treatment with four different antimicrobials. Since the antibiotic carbenicillin is only effective against fast-growing cells, only the logarithmic phase planktonic *P. aeruginosa* was eliminated significantly, with a small fraction of persisting cells able to survive the treatment. The inability of carbenicillin to kill biofilm cells was attributed to the supposition that the majority of cells in the biofilm were slow growing, and could therefore be compared to planktonic cells in stationary growth phase.

Unlike carbenicillin, the antibiotic ofloxacin is able to kill cells that are not actively growing. When tested against the cultures in each of the three growth phases, it proved to be effective against all three, although persister cells were observed in all three cases. The relative abundance of persister cells present in the stationary-phase planktonic cells were, however, markedly higher than those in the biofilm culture. The authors also found that a high density of cells positively influences the presence of persisters in a stationary-phase culture. Once the culture is diluted, the persisters tend to disappear, leading to speculation that quorum-sensing factors influence the formation of persisting cells.

Lewis (2000) furthermore suggested that the presence of persister cells is related to the programmed mechanism of cell death. The author argued that since most bacteria do not live in planktonic suspensions in nature, but rather in complex, multispecies consortia, it would be beneficial to the community as a whole if damaged cells could commit suicide. Lysis of damaged cells could provide additional nutrients to neighboring cells, as well as preventing

the damaged cells from depriving healthy bacteria from limited nutrients, while trying to repair their injuries. Persister cells might have an inoperative programmed cell death function, which could serve as a means of population survival. The persister cells are, however, not mutants. If the surviving persisters were cultured in fresh medium, the growth of the population would once again be as sensitive to the antimicrobial as the previous generation. The question remains as to how the bacteria decide whether to repair damage caused, commit suicide or in a few cases disable programmed cell death, when challenged by some potentially lethal factor.

The contention that persister cells are deficient in some mechanism of programmed cell death (Lewis, 2000) was disputed by Sufya *et al.* (2003). The authors showed that the level of persister cells was correlated to growth rate, where low nutrient concentrations resulted in slow growing populations of *E. coli* with higher percentages of persisting cells. In addition, the level of persistent cells was decreased by increased dosage concentrations of the antimicrobial. Since the amount of cells with a deficiency in programmed cell death exists independently of the concentration of antimicrobial applied, the theory proposed by Lewis (2000) does not seem to be able to account for low levels of persisting cells.

Sufya *et al.* (2003) examined the maximum specific growth rates of individual clones from batch cultures and found that the average doubling times of individual clones ranged between 45 and 500 minutes. Clones with a very low specific growth rates were shown to be unperturbed by the addition of antimicrobials. The presence of clones with such a wide range of growth rates, in a single batch culture or biofilm exposed to antimicrobial treatment, could explain the observed persistence of a subpopulation of cells.

In an unrelated study, the recalcitrance of *P. aeruginosa* infections in the lungs of cystic fibrosis patients has shed some light on the regulation of the conversion between susceptible and resistant bacteria within the same population, as well as possible phenotypic differences between the two forms (Drenkard and Ausubel, 2002). The prevalence of antibiotic resistant *P. aeruginosa* with altered colony phenotypes in sputum samples from patients suffering from cystic fibrosis has been well documented. One class of resistant colonies is smaller and rougher than the wild type, and exhibited an increased ability to form biofilms with distinct structures. A regulatory protein (PvrR) was found to control the conversion between antibiotic resistant and susceptible wild-type forms of *P. aeruginosa* in response to environmental signals. This observation has been compared to that of phenotypic variation, and it was proposed that the small amount of antibiotic resistant cells, with increased biofilm formation ability, ensure survival of the population during antibiotic treatment.

2.3.3.5 Substratum topography.

In a review of what was known about the influence of solid surfaces on bacterial activity at that time, Zobell (1943) concluded that seawater bacteria selected to attach to glass surfaces during low nutrient conditions. It was hypothesized that nutrients adsorbed to surfaces, thereby creating a favorable niche for bacterial development. In addition, the presence of the solid surfaces could slow diffusion of extracellular enzymes and hydrolyzed macromolecules away from the bacterial cell, allowing the cells to assimilate nutrients more efficiently.

While the presence of a surface provides the opportunity for attachment, an increase in the roughness of a surface may impede the penetration ability of antimicrobials. It was shown that *Salmonella enteritidis* colonize crevices in attachment surfaces and survive treatment with the antimicrobial trisodium phosphate (TSP). This allows the biofilm bacteria to proliferate, which further limits advective transport of the antimicrobial agent through the biofilm biomass and associated EPS. As a part of the same study, it was shown that biofilm age and the velocity of liquid flow could have an impact on the efficiency of antimicrobial treatment (Korber *et al.*, 1997).

2.3.3.6 Attachment-specific resistant phenotype.

A recent development within the field of biofilm related antimicrobial resistance involves the proposal of an attachment- or biofilm-specific resistant phenotype. The development of a biofilm requires changes in gene expression, whether it is induced by the presence of a surface, or by processes in the biofilm itself (Kuchma and O'Toole, 2000; Lewis, 2001; Stewart and Costerton, 2001; Gilbert *et al.*, 2002b). As discussed in Section 2.2., several genes and associated regulatory cascades that are involved in biofilm growth and development have been identified. The current search for genes involved in biofilm formation could reveal the existence of genes which, when induced upon attachment, provides increased protection against antimicrobial compounds.

Alternatively, a combination of all the mechanisms described above could provide a measure of protection when acting in combination, with each heightening the effect of the other. The inherently heterogeneous nature of biofilms, with respect to composition of the matrix, spatial organization and nutrient gradients, could also be held responsible. The presence of bacterial cells in various different physiological conditions throughout the biofilm represent a formidable target to an antimicrobial, especially when that antimicrobial has limited activity at single target sites. The application of an antimicrobial could fail even if only a few cells

within the biofilm survive (persisters), since these cells could theoretically multiply and serve as a source of re-infection.

2.3.4 Antimicrobial agents – Biocides.

Biocides are synthetic organic or inorganic molecules tasked with the prevention of microbial contamination and accompanying degradation. Biocides include a wide range of formulations, ranging from those containing metals, such as tin or copper as active compound, to organic heterocyclic molecules such as isothiazolones. These substances have been used for sanitization, disinfection, sterilization of surfaces, and the safeguarding of materials or processes from microbiological degradation for a century. This use has escalated since the public has been made more aware of hygiene (Mailliard, 2002; Chapman, 2003).

2.3.4.1 The mechanisms of biocide action.

It is generally accepted that, in contrast to antibiotics, biocides have more than one target site in the microbial cell, and that the concerted action at all of these sites results in cell death. Bacteriostatic actions may result from the application of lower concentrations of biocide and achieve only temporary cytoplasmic damage or loss of enzyme activity. Despite the widespread use of biocides, the precise mode of action is often not well understood (Mailliard, 2002).

Although biocides vary greatly with respect to composition, most target one or more structures within the microbe. Firstly, the outer envelope of mainly Gram-negative bacteria may serve as a target. This outer membrane consists of polysaccharides, proteins and porins that allow molecules to cross the membrane, while the structure itself can change over time as it is affected by environmental factors (Denyer and Mailliard, 2002).

Several biocides can act at the outer envelope, although it might not affect cellular viability. Glutaraldehyde is an example of such a biocide. Glutaraldehyde acts as a cross-linking agent, and by reacting with lipoproteins, it physically limits structures from functioning properly and also inhibits enzyme activity. Even though glutaraldehyde is very reactive and results in cell death when applied at high concentrations, it might not result in bacterial death when applied at low concentrations (Mailliard, 2002).

The second general target site is the cytoplasmic cell membrane. Several so-called membrane active agents are known, and these include phenols, alcohols, polymixins, parabens, biguanides and quaternary ammonium compounds (QACs). These compounds differ

considerably in structure and may therefore have different effects on the cell membrane. The physical disruption of the membrane may result in the loss of essential cytoplasmic compounds, such as proteins, nucleic acids, inorganic phosphates and potassium ions. The transmembrane proton motive force may also be dissipated, resulting in the uncoupling of oxidative phosphorylation and inhibition of membrane active transport (Denyer and Stewart, 1998; Mailliard, 2002).

The third target for disinfectants is the cytoplasm and its components. Antibacterial dyes, such as the acridines, interact physically with the nucleic acids and disrupt their function in the cell. Ozone and alkylating agents, such as formaldehyde, also target different chemical groups within the nucleic acids and disrupt the normal function of these essential molecules. The workings of other cytoplasmic constituents, such as ribosomes, may also be interrupted by highly reactive biocides (i.e. glutaraldehyde, peracetic acid, hydrogen peroxide and formaldehyde), even though they are not the principal targets (Mailliard, 2002).

The mechanisms of action of biocides can be divided into four categories (TABLE 2.2). Oxidising biocides, such as chlorine, interact rapidly with organic material via free radicals. The electrophilic biocides include organic biocides, such as formaldehyde and isothiazolones, as well as inorganic ions, with copper and silver as examples. These biocides could initiate the formation of intracellular free radicals, as well as react covalently with cellular nucleophiles, resulting in enzyme inactivation (Chapman, 2003).

TABLE 2.2: The mechanisms of action of biocides as summarized by Chapman (2003).

<table>
<tr><th colspan="4">Biocides</th></tr>
<tr><th colspan="2">Electrophiles</th><th colspan="2">Membrane Active</th></tr>
<tr><th>Oxidants</th><th>Electrophiles</th><th>Lytic</th><th>Protonophores</th></tr>
<tr><td>Halogens</td><td>Formaldehyde (FA)</td><td>Phenols</td><td>Parabens</td></tr>
<tr><td rowspan="3">Peroxy compounds</td><td>FA-releasers</td><td>Alcohols</td><td>Weak acids</td></tr>
<tr><td>Isothiazolones</td><td rowspan="3">Quaternary ammonium compounds</td><td>Pyrithione</td></tr>
<tr><td>Cu, Hg, Ag</td><td rowspan="2"></td></tr>
<tr><td>Bronopol</td></tr>
</table>

Quaternary ammonium compounds and alcohols act by destabilising cell membranes, leading to cell lysis (Mailliard, 2002; Chapman, 2003). The weak acids and pyrithione interfere with the ability of cell membranes to maintain a suitable pH balance, leading to a lower pH and disruption of metabolism within the cell.

2.3.4.2 Microbial resistance to biocides.

The extent of resistance developed by the bacterial cell towards biocides depends in part on the reactivity of the biocide, especially since most biocides have multiple target sites in the cell and the number of targets attacked depends on the reactivity of the biocide. Powerful oxidants can react with essentially all the organic molecules in the cell, while less reactive biocides, such as isothiazolones, can only react with strong nucleophiles (Chapman, 2003).

As is the case with antibiotics, microbes could gain resistance to biocides by altering the target site(s). The greater the number of targets, the more remote the possibility of altering enough target sites to confer resistance towards the biocide.

The microbes could, however, also achieve resistance by decreasing the number of potential target sites or through inactivation of the biocide. Mutations in the regulatory elements of certain genes could also produce resistant strains. The extensive extracellular polymeric matrix surrounding biofilms has been identified as a protective barrier against the oxidative biocide, chlorine (Chapman, 2003).

2.3.4.3 Examples of biocides used in industry.

2.3.4.3.1 Isothiazolones.

Isothiazolones react by oxidizing accessible cell membrane thiol groups, such as those found in cysteine and glutathione, to their disulphide adjuncts. Thiol residues are essential for the activity of several enzymes. The oxidation of these groups lead to an altered protein conformation and a disruption in its function, resulting in cell inhibition or even inactivation (Brözel and Cloete, 1994; Mailliard, 2002).

Isothiazolones have been shown to be effective against fungal as well as bacterial species, such as *P. aeruginosa* (Winder *et al.*, 2000). It has, however, been reported that some species show resistance against isothiazolones after prolonged exposure. Resistance towards isothiazolone has been induced in *P. aeruginosa* by incubating successive cultures with

increasing sub-inhibitory concentrations. Investigations revealed that a 35 kDa outer membrane protein was absent in cells resistant to the antimicrobial. The production of the protein was repressed within 24 hours of exposure to isothiazolone (Brözel and Cloete, 1994; Winder *et al.*, 2000). This observation resulted in the hypothesis that the membrane protein is responsible for the transport of isothiazolone into the bacterial cell (Brözel and Cloete, 1994).

Winder *et al.* (2000) found, in contrast to that of Brözel and Cloete (1994), that the membrane protein reappeared when resistant bacteria were re-cultured in biocide-free medium. They concluded that the gradual development of resistance was a function of phenotypic adaptation, rather than mutation, and this is supported by the reappearance of the membrane protein and biocide sensitivity when the pressure of biocide presence is alleviated. This observation supports the hypothesis that bacteria can develop resistance to antimicrobials when challenged with sub-inhibitory concentrations in their immediate environment.

2.3.4.3.2 Glutaraldehyde (or 1,5-pentanedial).

Glutaraldehyde is used extensively for disinfection and sterilization in such diverse industries as poultry farming, leather tanning, cosmetic production and in medical fields. It has a broad range of activity against bacteria, bacterial spores, fungi and even viruses (McDonnell and Russell, 1999). Laopaiboon *et al.* (2003) reported that glutaraldehyde (FIGURE 2.1) is routinely used to control microbial contamination in cooling water systems and is in all likelihood the most frequently used biocide in oilfield operations (Gardner and Stewart, 2002).

OH-----CCH2-CH2-CH2-C-----HO

FIGURE 2.1: The chemical structure of glutaraldehyde (McDonnell and Russell, 1999)

Grobe and Stewart (2000) tested the efficiency of glutaraldehyde against *P. aeruginosa* suspended cultures, as well as alginate bead-entrapped biofilms. A 2-log reduction in viable numbers of planktonic bacteria was observed after a 20 minute incubation with 590 mg/l glutaraldehyde, while the same loss of biofilm viability could only be achieved after a 600 minute reaction period.

The penetration of glutaraldehyde into the biofilm was found to be retarded, and this led to speculation that hindered diffusion of antimicrobials through biofilms contributed towards their reduced susceptibility when compared to that of planktonic cultures.

Subsequent studies on the effect of various biocides on artificial *P. aeruginosa* gel bead biofilms and suspended cultures involved the use of chlorine, glutaraldehyde, 2,2-dibromo-3-nitrilopionamide (DBNPA) and an alkyl dimethyl benzyl ammonium compound (ADBAC) (Grobe *et al.*, 2002).

As was the case previously, the biofilms were more resistant to killing with all four of the biocides tested, as compared to the planktonic cultures in the exponential growth phase. These authors observed that chlorine, glutaraldehyde and DBNPA reacted with and was neutralised by the alginate bead biofilms. This lead to the retarded diffusion of the biocides through the biofilm beads, and this was, in part, deemed responsible for the observed decrease in susceptibility.

The observed biofilm resistance to killing was linked to the dosage concentration of the biocide for all of the studied biocides. A lower dosage, applied for an increased period, was as effective as a higher concentration applied for a shorter time span. For example, a four times increase in glutaraldehyde concentration to 200 mg/l, applied for 34 minutes, was as effective as a 50 mg/l dosage for 650 minutes. This occurrence was observed only for the biofilm treated with glutaraldehyde, but not for the suspended cultures.

In another investigation, Gardner and Stewart (2002) examined the effect of between 50 and 500 mg/l of glutaraldehyde on sulphate-reducing bacterial biofilms. They found that the bacteria within biofilms were somewhat more resistant to glutaraldehyde treatment than planktonic bacteria. Sulphide production was used as an indication of the viability of the biomass. A 7-hour dosage of 50 mg/l glutaraldehyde suppressed sulphide production for 143 hours in planktonic cells. In comparison, a 7-hour treatment of the biofilm with 157 mg/l glutaraldehyde retarded the production of sulphide for 61 hours. No evidence of an adaptive response to glutaraldehyde by the biofilm was found after repeated treatments.

Importantly, they did find that glutaraldehyde reacted with, and was neutralized by the yeast extract in the Postgate C medium (Gardner and Stewart, 2002). As a result they replaced the growth media, flowing through the reactor, with 1.5% NaCl during the period of glutaraldehyde addition.

2.4. Physiological probes in combination with epifluorescence microscopy used to estimate bacterial viability.

The ability to quantify bacterial numbers is an important requirement in microbiology. The advent of fluorescent probes, in combination with epifluorescence microscopy, has to a degree succeeded in providing researchers with a means to estimate bacterial numbers. Direct counts have been used increasingly, instead of traditional spread plate methods, since the direct methods have been shown to significantly underestimate bacterial numbers by up to 10^4 times. The fluorescent directs counts also detect nonviable cells, as well as viable but non-culturable cells (VBNC), thereby providing a more complete picture (Kepner and Pratt, 1994).

The use of fluorescent direct counts became even more efficient with the introduction of polycarbonate Nucleopore filters, instead of cellulose acetate. Before black filters became commercially available, the filters were pre-stained with irgalan black to diminish background fluorescence (Hobbie *et al.*, 1977).

Samples are often preserved for subsequent analysis by epifluorescence microscopy. Adding aldehyde solutions, mainly 2% formaldehyde, usually effects preservation. Aldehydes function by cross-linking amine-groups of proteins and nucleic acids, thereby preserving the structure. Glutaraldehyde is widely used in microscopy as an effective fixative, preserving not only membranes, but also overall cellular integrity due to its cross-linking capabilities (Rogers, 1999). Glutaraldehyde has been used to preserve cell integrity at concentrations ranging from 1 to 2.5% (Kepner and Pratt, 1994).

Different fluorescent indicators respond to various cellular conditions, such as cell membrane permeability (Propidium iodide and 4', 6-Diamidino-2-phenylindole [DAPI], transmembrane electrochemical potential [Rhodamine 123], membrane integrity [Fluorescein diacetate]), reduction potential and enzymatic activity (Lloyd and Hayes 1995). While an indicator, measuring viability as a function of a particular cellular parameter, may prove to be useful under certain conditions, no indicator has been found to be universally applicable (Korber *et al.*, 1999). In addition, many of these indicators are not suitable for determining the viability of organisms obtained from the environment or cultured in the laboratory, due to the wide range of metabolic states present in different bacteria.

Plasmolysis of cells has been shown to provide an indication of cellular viability (Korber *et al.*, 1996). This physical indication of membrane integrity was used, in conjunction with

fluorescein and *Bac*Light™ viability probes, to successfully evaluate the viability of *S. enteritidis* biofilms following treatment with NaCl, heat and formalin.

The *Bac*Light™ LIVE/DEAD Viability Kit (Molecular Probes Inc., Oregon, USA) contains two fluorescent nucleic acid stain solutions, namely SYTO 9 and propidium iodide, and in combination determines whether a bacterial cell is viable or non-viable based on membrane integrity. SYTO 9 is able to cross bacterial cell membranes, and stain viable bacteria green, while the membrane-impermeable propidium iodide can only cross a compromised membrane, where it then overwhelms any green fluorescence to stain the cell red.

The manufacturers of the *Bac*Light™ kit have tested the reliability of the kit and are confident in the ability of the product to distinguish between live and dead bacteria. They do, however, mention that variable results have been found when assessing the viability of marine bacteria from environmental samples.

The application of the *Bac*Light™ viability kit for the enumeration of the viable and total bacterial count in drinking water was evaluated by Boulos *et al.* (1999). Acting on the manufacturer's suggestion of preserving stored samples by the addition of glutaraldehyde, they tested the effect of glutaraldehyde, added to a final concentration of 5%, on the total and viable counts of a known *E. coli* reference strain and an environmental isolate of *Citrobacter freundii*. A 24-hour storage period with glutaraldehyde did not have a significant impact on the total and viable counts of *C. freundii*. It did, however, decrease the viable to total bacterial count ratio of the *E. coli* reference strain to 24%, compared to the 63% of the sample stored without glutaraldehyde. Based on these results, the authors decided not to add glutaraldehyde to stored samples.

In the same study, viable and total bacterial enumeration was compared using *Bac*Light™, DAPI (4,6-di-amidino-2-phenylindole), CTC (5-cyano-2, 3-ditolyl tetrazolium) and AODC (Acridine orange direct count). The direct viable counts obtained with the *Bac*Light™ probes were higher than that of CTC reduction. For the total bacterial count, the *Bac*Light™ counts were equal to the acridine orange, which was, in turn, higher than the CTC / DAPI counterstaining and the CTC / SYTO 9 counterstaining.

In an effort to place these results into context, the authors made the following observations. Since the reduction of CTC is an indication of respiratory activity, it might be a more effective indication of cellular viability than that of membrane permeability. Combined with the possibility that correct functioning of the two *Bac*Light™ stains, SYTO 9 and propidium

iodide, might be restricted by a decline in membrane permeability and/or inadequate concentrations of the stains, thereby limiting detection. The physiological state, i.e. culture conditions, as well as growth rates of the bacteria may also affect the permeability of the membrane and thus the direct viable counts obtained.

To test the efficiency of *Bac*Light™ under adverse environmental conditions, batch cultures of bacteria were incubated with increasing concentrations of chlorine. The *Bac*Light™ total bacterial counts were unaffected by the chlorination, while an increase in chlorine concentration resulted in a decrease in the *Bac*Light™ viable counts. The total viable counts were, however, significantly higher (~1.2 log) than the traditional colony counts.

The authors concluded that *Bac*Light™ staining is not only reliable in terms of viable and total bacterial counts, but also results in far less background fluorescence than staining with acridine orange, for instance (Boulos *et al.*, 1999).

Chapter 3

MATERIALS AND METHODS

3.1 *In situ* cultivation of a natural biofilm community and evaluating the ability of attached and suspended microbes to survive antimicrobial treatment in a cooling water system.

A perspex cassette containing with vertically oriented, parallel glass slides (76 mm x 26 mm x 1 mm) were placed in the water collection tray of an air-conditioning cooling tower. The cooling tower water was treated with a commercial biocide (containing isothiazolone) at a concentration recommended by the supplier and/or anticorrosion agents (30-80 ppm) once a week.

After an incubation period of 1, 4, 7 and 14 days, respectively, three of the glass slides were removed from the holder and placed in separate bottles, along with 100 ml of water from the collection tray. Two of the bottles contained 1 part biocide to 10 000 parts of cooling water, while the remaining one contained only cooling water. The widely used commercial biocide consisted of glutaraldehyde and a blend of isothiazolones as active agents, at unknown concentrations. The biocide stock solution contains 9 to 12% of active concentration of the isothiazolones, with the ratio of glutaraldehyde to isothiazolone being three to one (personal communication, biocide supplier). The biocide is a broad-spectrum antimicrobial, used for controlling bacterial and fungal growth in pulp-, paper- and other related systems. The distributors also claim that the agent can reduce slime formation and microbiologically induced corrosion. All three bottles, each containing a glass slide and cooling water, were incubated for 5 hours in the cooling water tray.

The glass slides were thereafter washed with sterile water to remove loosely associated bacteria, and stained with 100 µl of *Bac*Light™ LIVE/DEAD viability probe (*Bac*Light™) for 15 minutes, in the dark. Unbound stain was removed with sterile water before mounting of samples for microscopic examination of the biofilm. Planktonic cells were studied by incubation of three millilitres of cooling water from each bottle with 100µl *Bac*Light™ probe for 20 minutes in the dark. The suspension was vacuum filtered through 0.22 µm, 47 mm Micron-PES, Polysulfone membrane filters (Osmonics Inc.) and mounted in *Bac*Light™ mounting oil. The stained samples were analysed using epiflourescence microscopy. A Nikon Eclipse E400 epifluorescence microscope equipped with a multipass filter set, appropriate for viewing DAPI, as well as excitation/barrier filter sets of 465-495/515-555nm (Texas Red), and 540-580/600-660nm (FITC) was used. A COHU high performance CCD

Camera (Model nr.4912-5010/0000) and a Nikon (Coolpix 9909) digital camera were used to capture the images.

The *Bac*Light™ Viability Kit contains two fluorescent nucleic acid stain solutions, namely SYTO 9 and propidium iodide, mixed in equal volumes of 2 µl (67 µM of Component A, and 60 mM of Component B) with 1 ml sterile dH_2O for staining. In combination, these two components determine whether a bacterial cell is viable or non-viable, based on membrane integrity. SYTO 9 is able to cross bacterial cell membranes, and stain viable bacteria green, while the membrane-impermeant propidium iodide can only cross compromised membranes to stain non-viable cells red. The red fluorescence of a non-viable cell overwhelms the green fluorescence, allowing separate images of red and green fluorescence to be generated using the filter sets. Separate green and red fluorescence emission images of the same microscopic field (n = 60 for the glass slides and n = 30 for the filters) were captured randomly across the entire surface area of the sample.

The images were analysed with ScionImage software (Scion Corporation). The total percentage area of the microscope field covered by viable (green) or non-viable (red) bacteria was determined using manually entered threshold values. The viable cells were expressed as a percentage of all the cells present in each field (Korber *et al.* 1996).

3.2 Isolation of bacteria from the cooling tower water.

Seven bacterial strains were isolated on $3g.l^{-1}$ Tryptone Soy Agar plates (TSA, Appendix) from the cooling tower water. Gram stains were performed on each of the isolates.

3.3 Cultivation of a biofilm community under laboratory conditions.

Perspex flowcells (FIGURE 3.1), covered with glass coverslips (no. 1 thickness, 75 x 50 mm) fixed with silicone adhesive, were attached with autoclaved silicone tubing (Inner diam. 1,6 mm, Outer diam. 3 mm) to a reservoir containing $3g.l^{-1}$ Tryptone Soy Broth (TSB) as growth medium. Each flowcell contained eight recessed flow chambers with an inner diameter of approximately 310 mm x 40 mm x 2.2 mm.

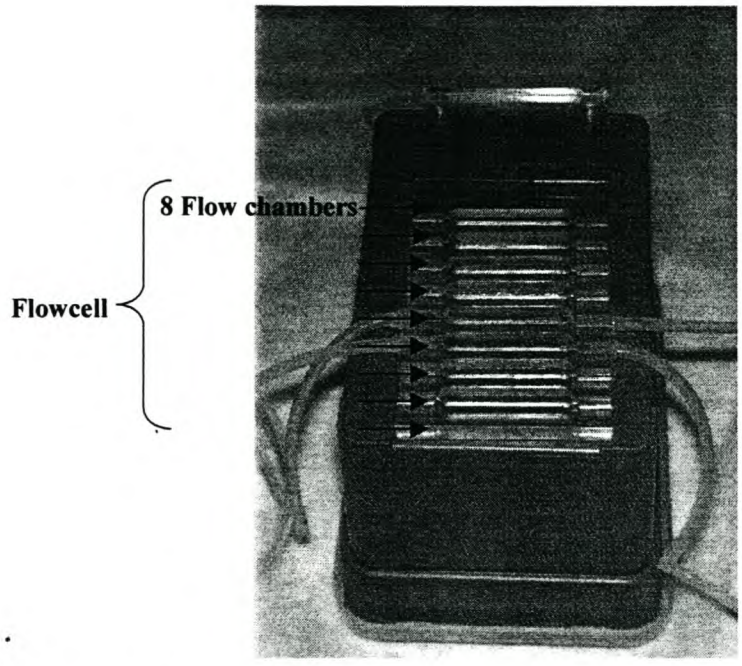


FIGURE 3.1: A perspex flowcell with eight recessed flow chambers. A glass coverslip is sealed onto the flowcell with silicone glue, and serves as the attachment surface (Wolfaardt *et al.*, 1994).

The flow chambers were sterilised with 3.5 % (v/v) Sodium hypochlorite for 20 minutes and rinsed with sterile growth media for at least 20 minutes before inoculation. One millilitre of a 10 ml 3 $g.l^{-1}$ TSB overnight culture of each of the seven microbial isolates, incubated at 30°C, was mixed aseptically and used as flowcell inoculum. Each flowcell chamber was inoculated aseptically with 200 µl of the mixed microbial community using a syringe and needle. A growth medium flow rate of 0.32 $ml.min^{-1}$ (19.2 $ml.h^{-1}$) was resumed after 20 minutes and maintained with a Watson Marlow 323S/318MC10 multichannel peristaltic pump at room temperature for 1 day, 4 days, 7 days and 14 days, respectively (FIGURE 3.2).

At each of the four time periods, the growth media irrigating two flow chambers was replaced with growth medium containing 1 part biocide to 100 parts of 3 $g.l^{-1}$ TSB. It was decided to increase the dosage concentration applied during the previous experiment (Section 3.1), to achieve a better discernable reduction in viability. After 5 hours of biocide treatment, the growth medium was reverted to 3 $g.l^{-1}$ TSB without biocide for the two treated flow chambers. Flowcell effluent from the two biocide treated and two untreated flow chambers were collected aseptically at each of the time periods.

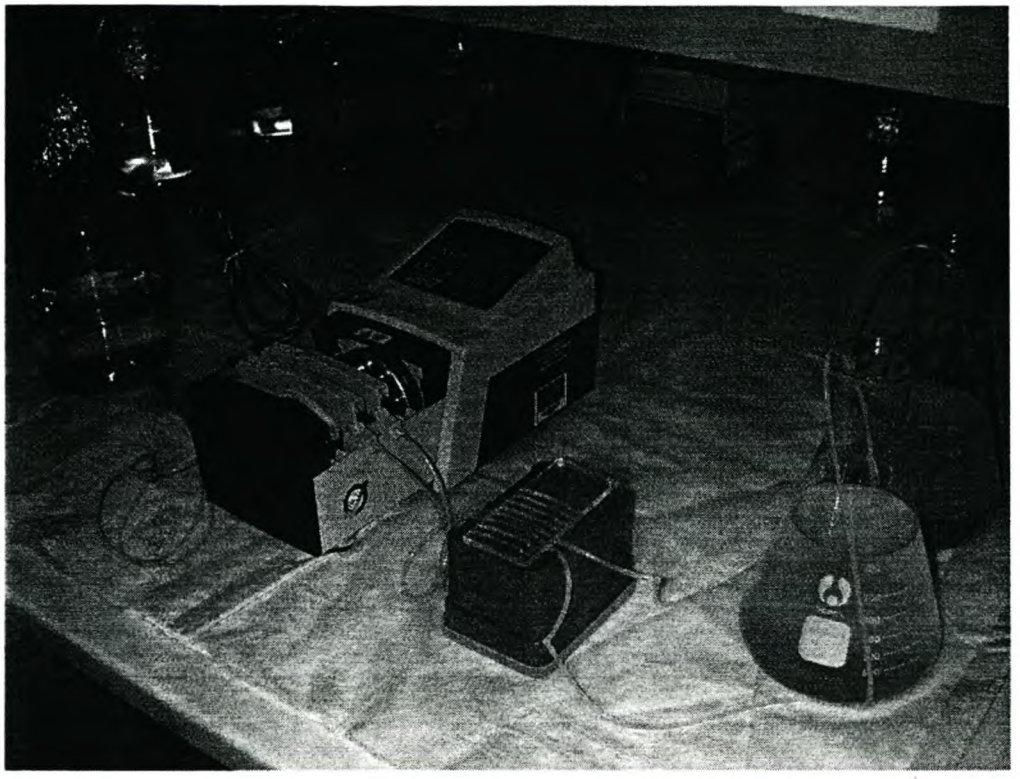


FIGURE 3.2: The typical continuous flow cultivation of biofilms in perspex flowcells. A reservoir, containing sterile growth media, is connected with silicone tubing to the peristaltic pump, flowcells and waste collection flasks.

Following collection, 1 ml of the effluent from each of the four chambers (two untreated and two biocide treated) was incubated with 50 µl *Bac*Light™ in the dark for 1 hour. After incubation the effluent was vacuum filtered through a 0.22 µm white polycarbonate filter (25 mm diam., Osmonics Inc.), mounted on a glass slide with mounting oil and covered with a glass coverslip (no. 1 thickness, 22 mm x 40 mm, Marienfeld) before visualisation using an epifluorescence microscopy as previously described. Images of thirty microscopic fields, randomly chosen across the entire filter surface, were captured and analysed.

The four biofilms (two untreated and two biocide treated) were also stained with 200 µl *Bac*Light™ for 15 minutes in darkness with arrested flow. Unbound *Bac*Light™ solution was washed out from the chamber with growth medium for 2 minutes before observing several microscope fields with the same epifluorescence microscope (n = 30). The images were analyzed using ScionImage software as previously described in Section 3.1.

Statistical analysis was performed to determine the optimal number of microscopic fields that need to be considered to attain the statistically representative percentage viability from each filter and/or flow chamber. The analysis method is described below.

All the values (i.e. percentage viability) obtained for each filter or flow cell chamber is arranged in order from smallest to largest, with the exception of the largest value, which is listed first. The mean of the largest and smallest value is determined and then the mean of the largest, smallest and then the second smallest value. This is continued until the mean has been determined for all of the values in the list.

$$\text{Mean} = \sum x_i / n$$

Where n is the number of values in the sample, and

x_i is any value in the sample

The standard deviation is calculated in the same manner as the mean, using the same values in each case.

$$\text{Standard deviation} = \sqrt{(\sum x_i - x)^2 / n}$$

Where x is the average of all the data points calculated up to that point in the list.

The coefficient of variation for each value in the series is calculated by dividing the standard deviation by the mean, which is then plotted against the cumulative sample size (the number of images in the series) in a scatter plot to determine the optimal sample size (FIGURE 3.3). The variability between measurements decreases as the number of analyzed fields increases, until a point is reached where the coefficient of variation no longer decreases (i.e. where the line reaches an approximate straight line, which then represents the optimal number of fields).

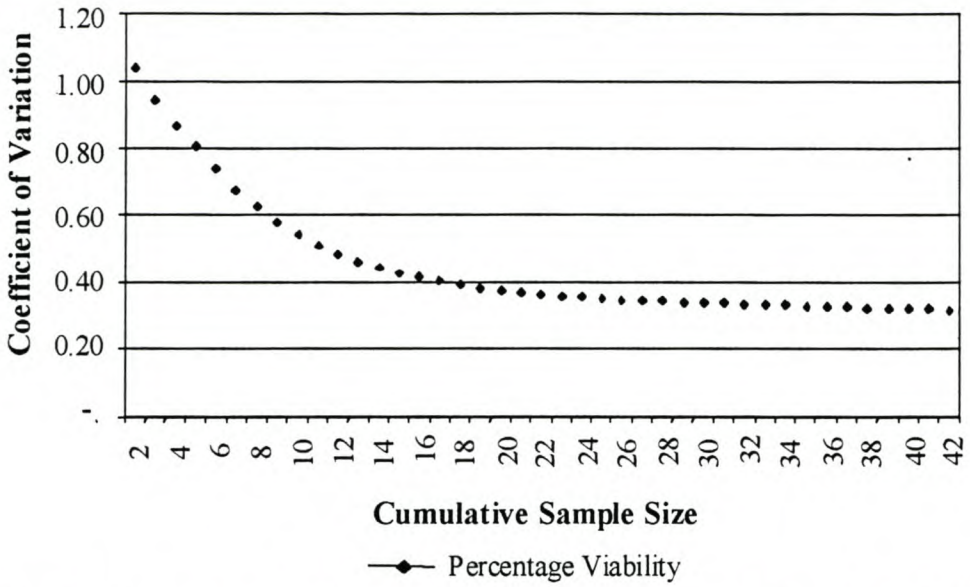


FIGURE 3.3: An example of a scatter plot of the percentage viability values obtained from various points across the attachment surface of a flowcell biofilm stained with *Bac*Light™ probes. The coefficient of variation is calculated as described above, and plotted against the cumulative sample size. The optimal sample size for this example is approximately 26.

3.4.1 Comparing the antimicrobial resistance of bacteria grown in a chemostat to biofilm bacteria cultivated in flowcells.

3.4.1.1 The growth rate of isolate CT07 in batch culture.

A prolific biofilm-forming isolate, CT07, was chosen for the subsequent experimental work. The specific growth rate of isolate CT07 was determined at room temperature. One millilitre of an overnight culture was inoculated into three flasks with 100 ml of 3 $g.l^{-1}$ TSB and incubated with agitation provided by a magnetic stirrer. Optical density readings at 600 nm were taken at one hour intervals over a period of 14 hours with a spectrophotometer (Ultrospec 3000, Pharmacia Biotech).

3.4.1.2 The specific growth rate of isolate CT07 in batch culture.

According to chemostat theory, at steady state the specific growth rate (μ) is equal to the dilution date (D) through the system. The dilution rate, in turn, is equal to the flow rate (F) per volume (V) of the system.

Therefore:

$D\ (h^{-1}) = F\ (ml.h^{-1}) / V\ (ml)$

Thus, in a steady state:

$D\ (h^{-1})$ or $\mu\ (h^{-1}) = F\ (ml.h^{-1}) / V\ (ml)$

The specific growth rate is determined graphically by plotting the increase in optical density against time. The greatest increase in optical density in one hour for isolate CT07 was determined from the graph and was used to calculate the specific growth rate (μ), as follows:

$$\mu\ (h^{-1}) = \ln (n_{t2}/n_{t1}) / (t_2-t_1)$$

Where n_{t1} is the initial optical density at time t_1 and n_{t2} is the optical density at time t_2.

Once the specific growth rate (or the maximum dilution rate) for isolate CT07 was known, the flow rate of the growth media through the chemostat was determined:

$$F\ (ml.h^{-1}) = \mu\ (h^{-1}) \times V\ (ml)$$

3.4.1.3 Determining the origin of the planktonic bacteria in the flowcell effluent.

According to chemostat theory, the maximum flow rate (F) at which planktonic dividing cells can be maintained without being washed out of the system, can be calculated by multiplying the dilution rate (D, equal to the specific growth rate, μ) with the volume of the vessel (V). If a dilution rate, greater than that of the specific growth rate of the bacteria, is applied to a continuous-flow system, such as a chemostat or flowcell, it would result in a washout of all planktonic bacteria, since they would be unable to replicate fast enough to remain within the system.

3.4.1.4 Comparing the ability of sessile, planktonic and flowcell effluent bacteria to survive antimicrobial treatment.

An aerated continuous-culture with a retention volume of 200 ml was used (FIGURE 3.4). The chemostat, containing 200 ml of sterile 3 $g.l^{-1}$ TSB, was inoculated with 1 ml of an overnight culture of isolate CT07. The culture was allowed to grow overnight at room temperature as a batch culture in the chemostat, with continuous stirring and aeration, before

introducing sterile 3 $g.l^{-1}$ TSB, with a peristaltic pump, at a flow rate below 58 $ml.h^{-1}$ for a period of 2 days.

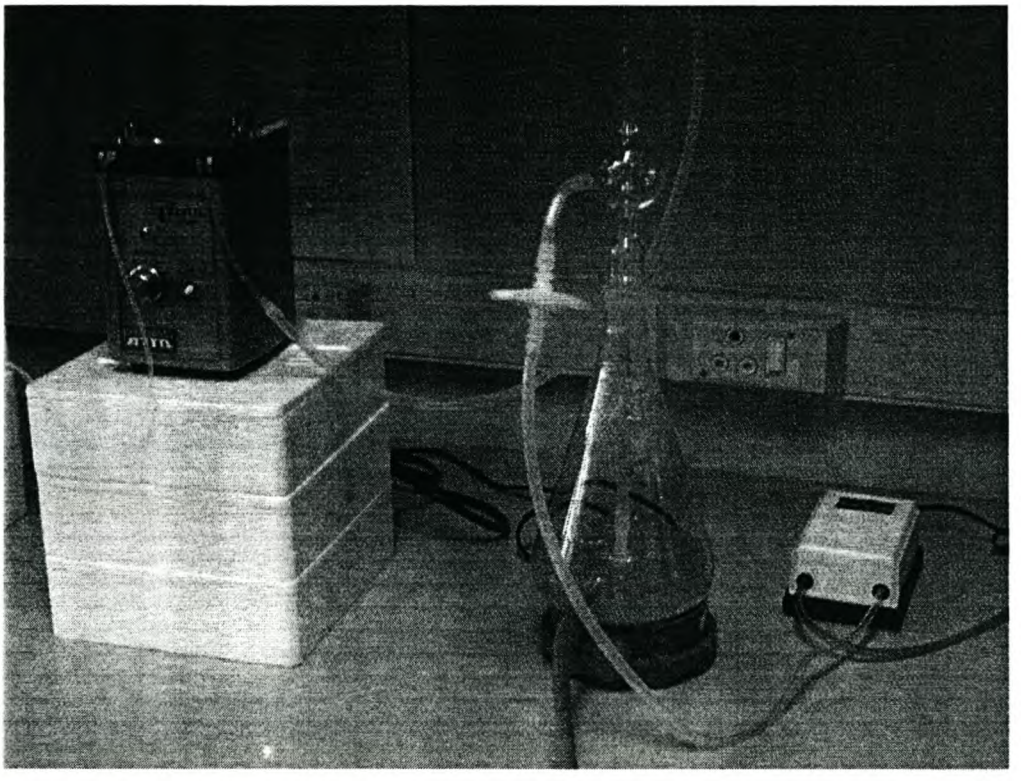


FIGURE 3.4: A planktonic population of CT07 was cultivated in a 200 ml glass continuous-culture. A single channel peristaltic pump provided the flow of sterile growth media from a reservoir, while air was fed into the system through a 0.47 μm venting filter from an aquarium air pump. A magnetic bar and stirrer allowed agitation of the liquid within the chemostat.

A multiple-channel perspex flowcell, connected to a reservoir containing 3 $g.l^{-1}$ TSB, was inoculated aseptically with an overnight culture of isolate CT07. The flowcell and connecting tubing were sterilized beforehand as previously described (Section 3.3). The bacteria were allowed to adhere for 1 hour, before commencing growth medium flow at a rate of 0.24 $ml.min^{-1}$ (or 14.5 $ml.h^{-1}$) with a Watson Marlow 205S peristaltic pump. Biofilm development was continued for 2 days at room temperature.

After the two day incubation period, the flowcell and chemostat reservoirs containing 3 $g.l^{-1}$ TSB as growth medium were replaced with 3 $g.l^{-1}$ TSB containing a 1 part biocide to 100 parts of TSB. A volume of biocide was also added to the chemostat vessel to achieve the same dilution. The volume of TSB in the connective tubing of the flowcell and chemostat

was purged until the tubing contained both TSB and biocide, before commencing flow. Biocide treatment was continued for 5 hours before sampling and staining.

Before the biocide treatment, 5 ml of the culture in the chemostat was aseptically removed and 5 ml of the effluent from each of the two flow chambers was collected. The sampling process was repeated after the biocide treatment. 50 µl *Bac*Light™ was added to one millilitre of each sample and incubated in the dark for a minimum of 1 hour. This was performed in duplicate. The samples were vacuum filtered onto 0.22 µm black, polycarbonate filters (Osmonics Inc.) and visualized with epifluorescence microscopy and the percentage of viable cells per microscope field determined as described in Section 3.3. Dilution series of the collected effluent were carried out and plated in duplicate on 3 $g.l^{-1}$ TSA plates. The plates were incubated for 4 days at room temperature before determining the colony forming units per ml (CFU/ml).

The biofilms were stained with 200 µl of *Bac*Light™ as previously described (Section 3.3), before determining the percentage of viability of the biofilm with epifluorescence microscopy.

3.5.1 Determining the relationship between culturability and viability of planktonic CT07 cells after biocide treatment using the Most Probable Number (MPN).

Two Erlenmeyer flasks, each containing 50 ml of 3 $g.l^{-1}$ TSB, were inoculated with 1 ml of an overnight culture of isolate CT07. The flasks were incubated at room temperature, with moderate shaking until the cultures reached an optical density of approximately 0.8 at 600 nm.

Once the desired stationary growth phase had been reached, 0.5 ml of the culture was incubated with 100 µl *Bac*Light™ for a minimum of 1 hour in the dark and filtered for epifluorescence microscopy and image analysis. Another 1 ml was used to determine the number of culturable cells per ml after serial dilution (undiluted to 10^8) in 9 ml Ringers solution and plating on 0.3 $g.l^{-1}$, 3 $g.l^{-1}$ and 30 $g.l^{-1}$ TSA plates. The plates were incubated at room temperature for 2 to 3 days.

One millilitre from each of the two flasks were centrifuged at high speed in a benchtop centrifuge (Biofuge, Hereaus), the supernatant discarded and the cell pellet resuspended in 1 ml of 3 $g.l^{-1}$ TSB. The harvested cells were diluted separately (10^2 to 10^8), in triplicate test

tubes containing either 0.3 $g.l^{-1}$, 3 $g.l^{-1}$ or 30 $g.l^{-1}$ TSB, and incubated at room temperature for 2 to 3 days to determine the Most Probable Number of bacteria present.

The commercial biocide was added to the remaining culture, at 1 part biocide to 100 parts growth media and incubated for 5 hours at room temperature, with shaking. Thereafter the sampling was repeated with image analysis to determine the percentage viable cells after dosage and dilution and plating to determine culturable cells per ml and MPN. One millilitre of each of the two cultures was centrifuged at high speed and washed with 1 ml 3 $g.l^{-1}$ TSB, to remove residual biocide, diluted and incubated as described above.

3.5.2 Determining the relationship between culturability and viability of CT07 biofilms after biocide treatment.

Biofilms of CT07 were cultured on glass slides in a Pedersen continuous-flow device for a 5 day period (Pedersen, 1982). The device and connective tubing were sterilized with 3.5 % (v/v) Sodium Hypochlorite for 5 hours. Residual solution was washed out with sterile distilled H_2O overnight. Before inoculation the dH_2O was replaced with sterile 3 $g.l^{-1}$ TSB pumped from a reservoir containing 11 litres of the growth medium by means of a Watson Marlow peristaltic pump. The outflow was circulated back to the reservoir. The system was inoculated with 5 ml of an overnight culture with arrested growth media flow. One hour was allowed for attachment before the flow was resumed at 96.2 $ml.min^{-1}$.

At the end of the biofilm cultivation period, the 11 L, 3 $g.l^{-1}$ TSB reservoir was replaced with 1L of 3 $g.l^{-1}$ TSB containing the commercial biocide at 1 part biocide to 100 parts TSB for 5 hours. Thereafter, six glass slides were removed and gently washed with dH_2O. Sterile cotton swabs were used to remove attached cells from each of three slides. Swabs of half of each of the slides were placed in either 500 µl or 1 ml of sterile Ringers solution and briefly vortexed. One half of each slide was used for determining the viability after biocide treatment by incubating 500 µl with 100 µl *Bac*Light™ for 60 minutes in the dark and immobilization on 0.2 µm black polycarbonate filters (Osmonics Inc.) and image analysis as described in Section 3.3. The biofilm biomass from the other half of each slide was diluted in 1 ml sterile Ringers, plated on 3 $g.l^{-1}$ TSA and incubated at room temperature for 3 days. The attached cells on the three remaining slides were visualised with 200 µl *Bac*Light™, followed by incubation for 15 minutes in the dark. Approximately 30 microscope fields were considered from each slide to attain a statistically representative number. An additional three glass slides were removed before antimicrobial treatment to determine the CFU/ml of untreated biofilms as described.

3.6.1 Measuring protease activity of the isolates CT01 to CT07.

The reliability of the various techniques using fluorescent probes to estimate cellular viability has been questioned and alternatives, such as the possible difference in protease activity between viable and compromised bacteria were explored.

Isolates CT01 to 07 were initially assayed separately and together for the production of proteases using a spot test on Luria Bertani Broth Skim milk plates (Appendix). Each of the organisms were grown in 10 ml 3 $g.l^{-1}$ TSB overnight at 30°C before spotting 15 µl of each culture on a Luria Bertani Broth Skim milk plate. Proteinase K was used as a positive control. The plates were incubated for 2 to 3 days at 30°C.

Thereafter, an adaptation of the method of Long *et al.* (1981) as described by Deane *et. al.* (1987) using the synthetic substrate azocasein (Sigma-Aldrich), was used to detect protease production.

Each of the seven isolates was cultured for 24 hours in 3 $g.l^{-1}$ TSB at 30°C. Thereafter 250µl of the culture supernatant was mixed with 250 µl azocasein solution (Appendix) and placed on ice before incubation in a 37°C water bath for 30 minutes. The reaction was stopped with the addition of 500 µl ice-cold 10 % Trichloro-acetic acid (TCA, Sigma-Aldrich). After 30 minutes on ice, the tube was centrifuged for 2 minutes in a Biofuge microfuge at maximum speed to remove the precipitate. The assay supernatant was mixed with an equal volume of 0.5 M NaOH, and the absorbance read at 440 nm. Each assay tube had its own blank, in which the assay reaction was stopped by the addition of the 500 µl 10% TCA before incubation in the water bath. One unit of protease activity is defined as the amount of enzyme that gives an increase in absorbance of 0.1 at 440 nm in 30 minutes at 37°C. The process was repeated with 48-hour-old cultures of each of the organisms.

3.6.2 Determining the protease activity of flowcell effluent.

The effluent from each of the four chambers (Section 3.3) after four, seven and fourteen days was also used to assay for protease production using the modified method of Long et. al. (1981) as previously described in Section 3.6.1.

3.7 Antimicrobial resistance of batch cultures of isolate CT07 in the three different growth phases.

Erlenmeyer flasks containing 50 ml of 3 $g.l^{-1}$ TSB, were inoculated with 1 ml of an overnight culture of isolate CT07. The flasks were incubated at room temperature, with moderate shaking for different time periods. The optical density (OD) of the cultures was measured at 600 nm to determine when the bacteria had reached each of the three growth phases. For the lag phase, an OD_{600nm} of below 0,060 was chosen. Two readings between 0.100 and 0.690 were selected for the exponential phase and a reading greater than 0.780 for the stationary phase. This was performed in triplicate for each growth phase.

Once the desired growth phase had been reached, 0.5 ml of the culture was incubated with 100 µl *Bac*Light™ for a minimum of 1 hour in the dark and filtered for epifluorescence and image analysis as described in Section 3.3. Another 1 ml was used to determine the culturable amount of cells per ml after serial dilution (10^2 to 10^8) in 9 ml Ringers solution (Merck) and plating on 3 $g.l^{-1}$ TSA plates. The commercial biocide was added to the remaining culture, at a 1 part biocide to 100 parts 3 $g.l^{-1}$ TSB and incubated for 5 hours at room temperature, with shaking. Thereafter the sampling was repeated to determine the percentage viability and culturable amount of cells.

3.8 The specific growth rate of isolate CT07 at a surface.

Prior to inoculation with 1 ml of an overnight culture of isolate CT07, a flowcell was prepared and sterilized as described previously (Section 3.3). The bacteria were allowed to attach for a period of 30 minutes, before 3 $g.l^{-1}$ TSB medium flow was resumed at a rate of 0.24 $ml.min^{-1}$ (or 14.5 $ml.h^{-1}$) using a Watson-Marlow 205S peristaltic pump. The biofilm was allowed to develop for a period of 12 hours before the flowcell was secured on the platform of a Nikon microscope. The attachment of individual bacteria and subsequent microcolony formation was documented by light microscopy and images capture at 600X magnification at 30 minute intervals. The above experiment was duplicated.

Another approach involving an Optical Large Area Photometer (OLAPH, Patent pending) was used to quantify the development of a biofilm. The device continually monitors the accumulation of biomass at the surface of a double plated Plexiglass flowcell, with an internal volume of approximately 12 ml. Light is passed through the vertically orientated flowcell, while a sensor records the forward scattered light from the biofilm cultivated in the flowcell. As the bacteria attach and start to develop a three-dimensional biofilm within the flowcell, the

light intensity reaching the sensor changes and the biofilm development is recorded at regular intervals by a program run in LabView Student Edition, Version 3.1 (National Instruments).

The flowcell and tubing was sterilized overnight with commercially available 3.5 % Sodium Hypochlorite. The rectangular flowcell was oriented vertically, with the inflow at the top and outflow at the bottom. Flow was provided with a Watson-Marlow 502S peristaltic pump. The sterilising solution was purged with 3 $g.l^{-1}$ TSB for one hour before inoculation with 4,5 ml of an overnight culture of isolate CT07. The cells were allowed to adhere for 30 minutes, with flow arrested before resuming a flow rate of 0.24 $ml.min^{-1}$ (or 14.5 $ml.h^{-1}$) for approximately 6 days at room temperature. The changes in light emission were recorded at one-minute intervals. This was duplicated.

Chapter 4

RESULTS and DISCUSSION

4.1 *In situ* cultivation of a natural microbial biofilm community and evaluation of the ability of attached and suspended microbes to survive antimicrobial treatment.

It has been extensively reported that organisms in the biofilm mode of growth are more resistant to antimicrobial treatment than free-floating organisms. This is widely regarded as the rule, rather than the exception for most attached populations, and has been the focus of numerous studies involving antimicrobial resistance in medical and industrial situations (Vrany *et al.*, 1997; Broon *et al.*, 2000; Walters *et al.*, 2003).

To investigate the reported increased resistance of biofilms towards antimicrobial agents, it was first necessary to determine whether this was true for the microbial community in the environment under investigation. The hypothesis tested was as follows: An attached microbial community cultivated in a natural environment, with a history of exposure to antimicrobials, is more resistant to killing by antimicrobials than the equivalent planktonic population.

The ability of free-floating organisms to survive the adverse conditions was compared to that of biofilm communities of different ages (FIGURE 4.1). Determining the reduction in the percentage of viable bacteria after biocide dosage, using *Bac*Light™ viability probes and epifluorescence microscopy, was more complex than anticipated. The viability of day-old biofilm bacteria, as well as their planktonic counterparts, seemed to increase after incubation with the biocide. The data obtained for a four-day-old biofilm corroborates the reported increased resistance of attached bacteria compared to that of free-floating bacteria, but this is disputed by data obtained for both a seven and fourteen-day-old biofilm.

Statistical analysis revealed large standard deviations when determining the average percentage viability at different points across the filter and glass slide surfaces and between the duplicate glass slides. The inherent heterogeneous three-dimensional structure of biofilms could be responsible for this observation. Scanning confocal laser microscopy (SCLM) studies of natural biofilm communities have shown that the spatial arrangement of bacteria within biofilms can be extremely diverse. In addition, the presence of various extrapolymeric substances and pores or channels permeating the biofilm can contribute to the complexity (Lawrence *et al.*, 1996).

Korber *et al.* (1994) also reported an increase in the standard deviation when measuring the mean biofilm depth of *P. fluorescens* biofilms and attributed it to the presence of channels, which penetrated to the base of the biofilm.

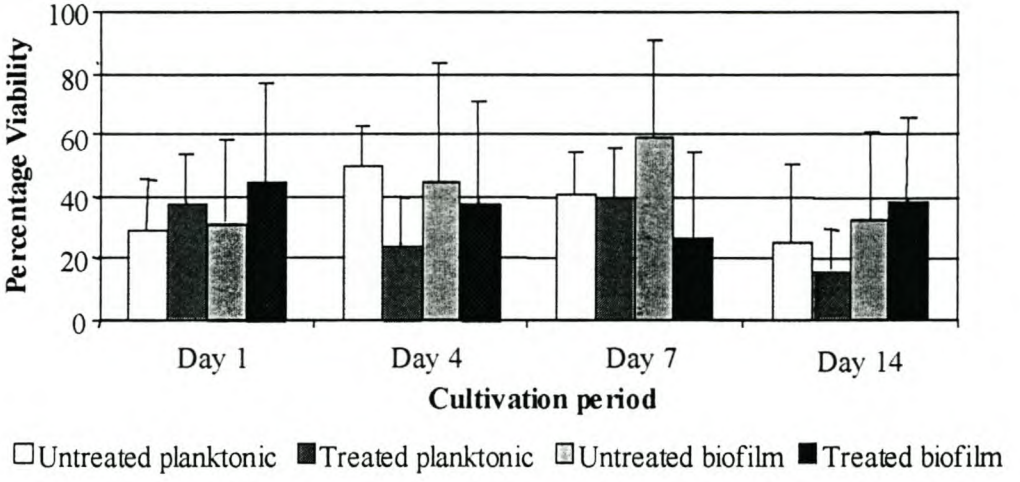


FIGURE 4.1: Biofilms were cultivated on glass for different time periods in the water collection tray of an air-conditioning cooling tower, before subjecting both the biofilm (on the glass slides) and planktonic organisms (in the water) to antimicrobial treatment in the form of 1 part biocide, to 10 000 parts of cooling water. After treatment, the percentage viable bacteria were determined using *Bac*Light™ fluorescent probes and epifluorescence microscopy. An untreated biofilm on a glass slide and cooling water, were incubated along with the treated samples to provide an indication of the viability before treatment. All tests were performed in duplicate.

In response to the observed variability of the viability data, it was decided to attempt a more statistical approach and determine the optimal number of microscopic fields that have to be considered to attain a representative analysis across a glass slide, filter or flowcell attachment surface (Section 3.3). Based on the results obtained, the null hypothesis could not be rejected, and it was decided to test it again after refining the experimental design.

4.2 The isolation of organisms from cooling water.

The above experiment was repeated under defined laboratory conditions, so that a comparison could be made between the survival ability of natural biofilm and planktonic communities after incubation with the biocide. To this end, it was decided to isolate bacterial species, based on colony morphotype, from water in the air-conditioning cooling tower (TABLE 4.1). Isolations were done on 3 $g.l^{-1}$ TSA plates, incubated at room temperature. Freeze cultures were made in 40 % glycerol and kept at −18°C. Gram stains were performed to determine cell morphology followed by sequencing of their 16S rDNA.

TABLE 4.1: Seven bacterial strains were isolated from air-conditioning cooling water and designated CT01 to 07.

Isolate	Colony morphology on 3 $g.l^{-1}$ TSA plates	Colony colour	Gram stain	Cell morphology
CT01	Non-transparent Shiny, grainy inside Smooth edge	White	Positive	Ellips Single or chains
CT02	Non-transparent Shiny Smooth edge	Bright yellow	Positive	Elongated Ellips-shaped
CT03	Semi-transparent Shiny inside with grainy edges	Creamy white	Negative	Single or chains of elliptical cells
CT04	Non-transparent Shiny Smooth edges	Orange	Negative	Diplo-ellips
C05	Semi-transparent Grainy Grainy edges	White	Negative	Ellips / round Single cells
CT06	Non-transparent Shiny	Pink	Negative	Rods
CT07	Semi-transparent Shiny Raised edges with lowered centre	Cream	Negative	Rods

4.3 The cultivation of a biofilm community under laboratory conditions.

A multi-species community, consisting of seven bacterial isolates (TABLE 4.1), isolated from the cooling tower water, was used as inoculum for the flowcells. The bacterial communities in the flowcells were exposed to antimicrobial treatment, in the form of a commercial biocide, at an increased concentration of 1 part biocide to 100 parts of 3 $g.l^{-1}$ TSB. The response of attached bacteria to the treatment was compared to that of the free-floating bacteria within the flowcell effluent (FIGURE 4.2).

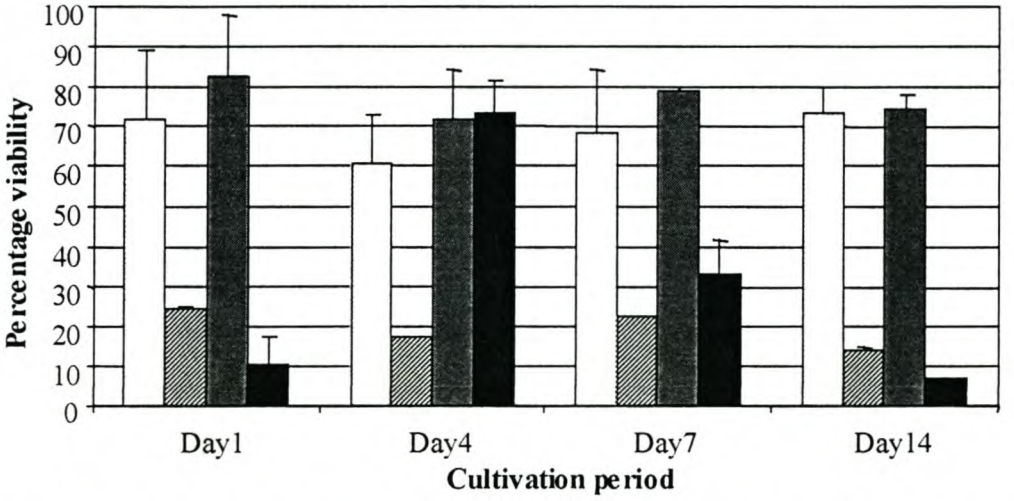


FIGURE 4.2: Biofilms were cultivated in perspex flowcells with 3 $g.l^{-1}$ TSB as growth media at a flow rate of 0.32 $ml.min^{-1}$. After one, four, seven and fourteen days, respectively, two flow channels were treated with 3 $g.l^{-1}$ TSB containing the commercial biocide for a period of five hours. The biofilm and sampled effluent from two treated and two untreated channels were stained with *Bac*Light™ to determine the percentage viable cells using epifluorescence microscopy and image analysis.

As with the previous experiment, no conclusive results could be drawn with regards to the response of attached and dispersed bacteria to biocide treatment even though an effort was made to include a statistically correct amount of observations per flow chamber and filter.

The inherent variability between duplicate flow chambers and filters once again proved to be significant. The inconsistent data led to speculation about the origin of the bacteria within the flowcell effluent. Two possibilities were proposed to account for the presence of bacterial cells in the effluent, namely that (1) a replicating community of planktonic bacteria exists independently of the attached biofilm in the flow chamber and (2) the bacteria are released from the biofilm by natural detachment processes or as a result of treatment with an antimicrobial.

The ability of attached bacteria to survive in the presence of antimicrobials, when free-floating bacteria could not has been attributed, amongst other mechanisms, to the existence of a biofilm-specific phenotype (Kuchma and O'Toole, 2000; Lewis, 2001; Stewart and Costerton, 2001; Gilbert *et al.*, 2002b). The expression of genes, thought to be unique to the biofilm mode of existence, could explain the greater resistance exhibited by sessile microbes to antimicrobial treatment. Points of contention in this regard include the induction of this biofilm specific gene expression pattern and the rate whereby bacteria are able to respond genetically to the presence of a surface. If differential gene expression patterns do exist, the natural shedding of bacteria from the biofilm most likely also requires a change in gene expression. The time required to alternate between the attached and planktonic phenotype, once the bacteria are released from the biofilm, could point toward the presence of a 'biofilm-memory'. The impact of such a gradual change in gene expression on the ability of the microbe to survive antimicrobial treatment then becomes of interest.

In order to test a secondary hypothesis, namely that the bacteria present in the flowcell effluent exhibited antimicrobial susceptibility similar to that of truly planktonic bacteria (i.e. that the daughter cells released from the biofilm have a gene expression pattern similar to that of planktonic cells), the ability of a chemostat-derived planktonic population to survive biocide treatment was compared to that of an attached community in a flow chamber and microbes in the effluent from these chambers.

4.4.1 Comparing the antimicrobial resistance of bacteria grown in a chemostat to biofilm bacteria cultivated in flowcells.

In order to test the hypothesis, it was decided to simplify the experiment by working with a single isolate. To this end, the prolific biofilm-forming isolate CT07 was chosen as model

organism. Sequencing of the 16S rDNA of isolate CT07 revealed a 99 % nucleotide identity (1489-bp / 1495-bp) to *Pseudomonas* sp. AEBL3 (AY 247063) when a comparison search was performed using the gapped-BLAST program at the National Center for Biotechnology Information (NCBI) [http://www.ncbi.nlm.nih.gov/BLAST/] (Altschul *et al.*, 1997).

4.4.1.1 The growth rate of isolate CT07 in batch culture.

To determine the maximal dilution rate in the chemostat, the specific growth rate of isolate CT07 was determined at room temperature in batch culture (FIGURE 4.3).

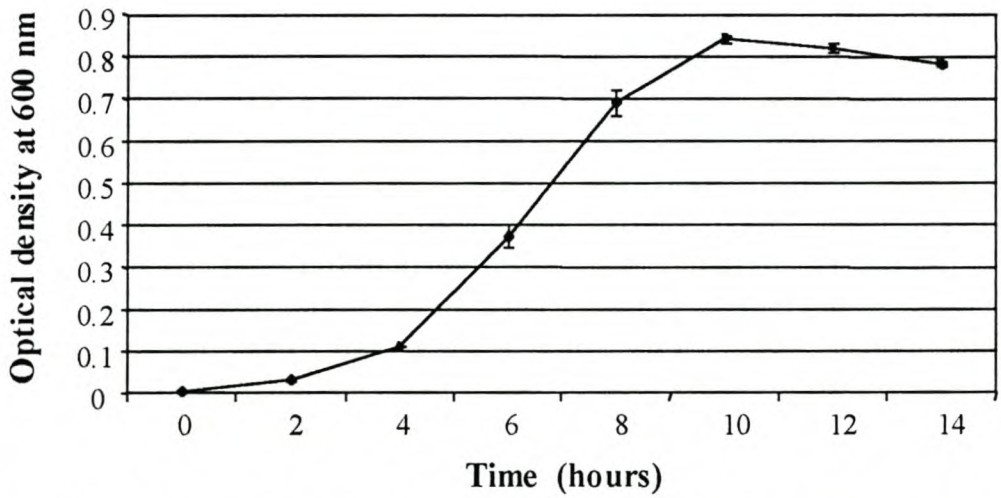


FIGURE 4.3: The growth rate of isolate CT07 as determined at room temperature (23°C ± 2°C) in a batch culture containing 3 $g.l^{-1}$ TSB. The specific growth rate (μ) was determined as 0.62 h^{-1} as described in Section 3.4.1.2. This was performed in triplicate.

4.4.1.2 Comparing the antimicrobial resistance of bacteria grown in a chemostat to biofilm bacteria cultivated in flowcells.

The comparison of the ability of the chemostat derived planktonic cells and biofilm bacteria to survive antimicrobial treatment as determined with *Bac*Light™ viability probes, revealed that the biofilm population was better able to survive treatment with an antimicrobial. The viability of control and treated biofilms remained virtually unchanged, with a slight reduction from 99.6 % to 96.9 % (FIGURES 4.4 and 4.5).

The chemostat derived cells were more susceptible to biocide treatment as the percentage of viable bacteria declined from 90.5 % to 58.0 %, while the viability of the flowcell effluent bacteria decreased substantially from 73.0 % to only 9.7 %.

This observation corresponds to the wealth of literature suggesting that the biofilm mode of growth is more resistant to killing by antimicrobials than the planktonic one. It is, however, important to note that the difference in the decrease in viability after antimicrobial treatment between the two populations is not statistically significant. The bacteria within the flowcell effluent are more susceptible to treatment with a biocide, compared to that of the biofilm (P = 0.043) and more susceptible to that of the chemostat bacteria, although not statistically significant.

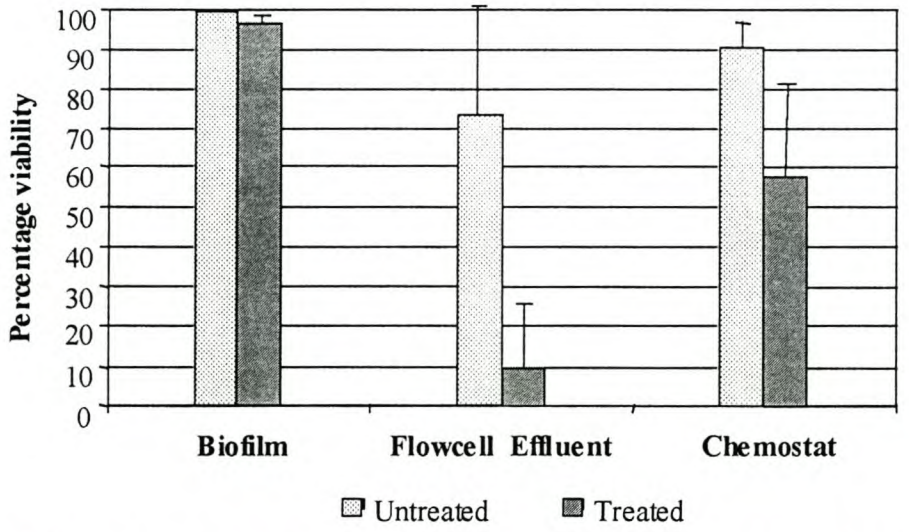
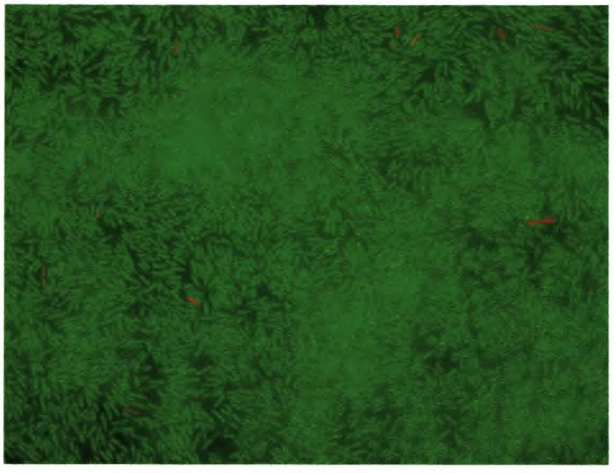


FIGURE 4.4: Comparison of the viability of control and biocide treated cells as determined with *Bac*Light™ viability staining. The reduction in the percentage viable cells, as a result of biocide treatment, was only significant when the biofilm and flowcell effluent results were compared using one-way analysis of variance (ANOVA, STATISTICA, Version 6.0, StatSoft) (P = 0.05). The experiment was repeated four times.

A:



B:

FIGURE 4.5: Photomicrographs of CT07 biofilms stained with *Bac*Light™ and visualized with epifluorescence microscopy. The biofilms were cultivated in flowcells for 2 days, with 3 $g.l^{-1}$ TSB. A: The untreated biofilm of CT07. B: The biofilm was treated with 1 part commercial biocide to 100 parts 3 $g.l^{-1}$ TSB for 5 hours.

In addition to determining the viability of the different cultures, the numbers of culturable cells in the flow chamber and chemostat effluent prior to, and following, incubation with the biocide were also assessed. No bacterial cells sampled from the flow chamber effluent were able to grow on 3 $g.l^{-1}$ TSA plates after treatment, while the CFU/ml in the chemostat decreased from 1.2×10^9 to 6.6×10^4 per ml as a result of treatment (FIGURE 4.6). Although this represents a 99.9% decline in the culturable amount of bacteria, there was a substantial number of surviving bacteria and this could still present a notable problem in industrial systems in terms of biofouling control. The observed trend in the loss of culturability corroborated the reduction in viability as determined with the *Bac*Light™ viability probes, but the difference in the percentage decrease in number is difficult to explain.

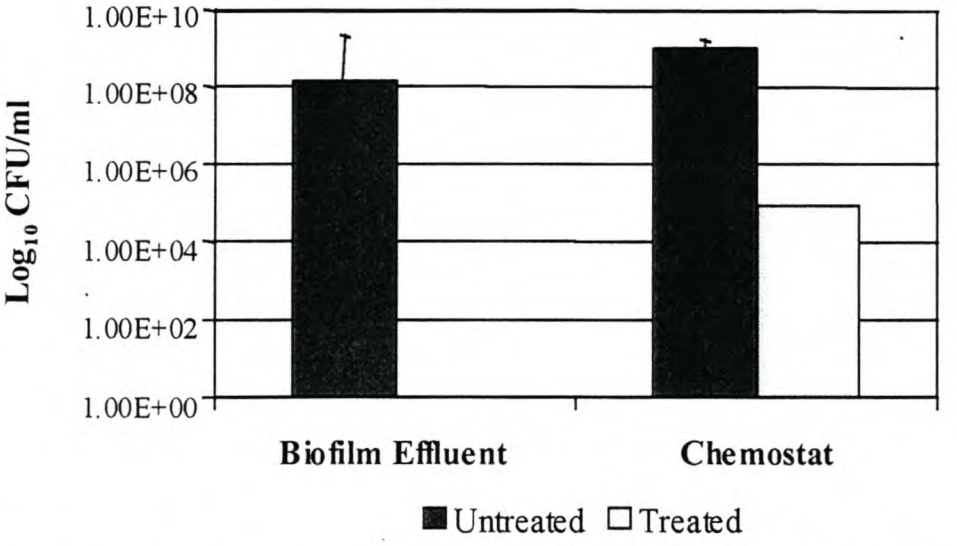


FIGURE 4.6: The cell numbers of untreated and treated chemostat and flowcell effluent were determined to evaluate the killing efficiency of the biocide, and compared to the reduction in viability as established with the *Bac*Light™ viability probes. No colony forming units per millilitre from the flowcell effluent were detectable after treatment with the biocide.

The estimation of bacterial viability has traditionally been achieved through culture-dependent techniques, such as plate counting. This technique relies on culturable bacteria with the ability to divide to produce visible colonies. The presence of viable but non-culturable bacteria (VBNC) could provide an explanation for the difference in the amount of viable cells detected with *Bac*Light™ (FIGURE 4.4), compared to that cultured on laboratory media (FIGURE 4.6). Gunasekera *et al.* (2002) reported that direct viable counts of pasteurized milk, as determined with *Bac*Light™, were significantly higher ($P = 0.05$) than conventional cell counts.

A VBNC state could allow the bacteria to persist during adverse environmental conditions by decreasing their metabolic activity. One of the hallmarks of this state is the inability of the microbes to reproduce and as such, they become unculturable using traditional methods such as plating on rich growth media. These bacteria may, however, be detected by culture independent techniques, such as viability stains (*Bac*Light™) as well as Reverse Transcriptase-PCR (RT-PCR) (Gunasekera *et al.*, 2002; Heim *et al.*, 2002). While the VBNC state can be viewed primarily as a survival strategy, it could also indicate that the

bacteria are incapable of dividing due to injury to some part of the cell. These bacteria may become culturable again under subsequent favourable environmental conditions.

In addition to the presence of a VBNC state, another explanation for the difference in viability and culturability data could be attributed to treatment with a biocide containing glutaraldehyde. Since the mode of action of glutaraldehyde involves the cross-linking of components, especially proteins, in the outer membranes of Gram-negative bacterial cells, it might influence the ability of one of the *Bac*Light™ components to penetrate the bacterial cell. Propidium iodide can only enter bacterial cells with compromised cell membranes to stain the nucleic acids red. If glutaraldehyde induces substantial conformational changes in the membrane structure, thereby preventing the penetration of propidium iodide into injured or killed cells; the number of bacteria adversely affected by antimicrobial treatment would be underestimated. Some have also suggested that glutaraldehyde might have an additional target in the cell membrane, since it prevents the lysis of spheroplasts or protoplasts when placed in hypotonic solutions (McDonnell and Russell, 1999). This suspicion remains to be confirmed, but could provide evidence for the reduced efficiency of one of the *Bac*Light™ components.

This disparity between the amount of viable cells and the number of CFU/ml present after antimicrobial treatment obtained was a cause for concern. We attempted to address this by determining the ability of treated planktonic bacteria to regrow after incubation with the biocide in a liquid culture using the three-tube Most Probable Number (MPN) technique, as well as removing untreated and treated biofilms from glass surfaces and determining the surviving CFU/ml as well as the percentage viable cells using *Bac*Light™ viability probes (Section 4.5.1 and 4.5.2).

The secondary objective of this experiment was to test the hypothesis that bacteria in the flowcell effluent exhibits similar resistance profiles to that of planktonic bacteria. The viability and culturability data disputed this hypothesis. The bacterial cells in the effluent were significantly more susceptible to antimicrobial treatment compared to the biofilm (95 % confidence level), indicating that a third phenotype, in terms of the ability to resist antimicrobial treatment, has been identified.

While the total amount of cells present at the attachment surface declined only slightly because of biocide treatment, the number of cells present in the effluent from the flowcell declined substantially (FIGURE 4.7).

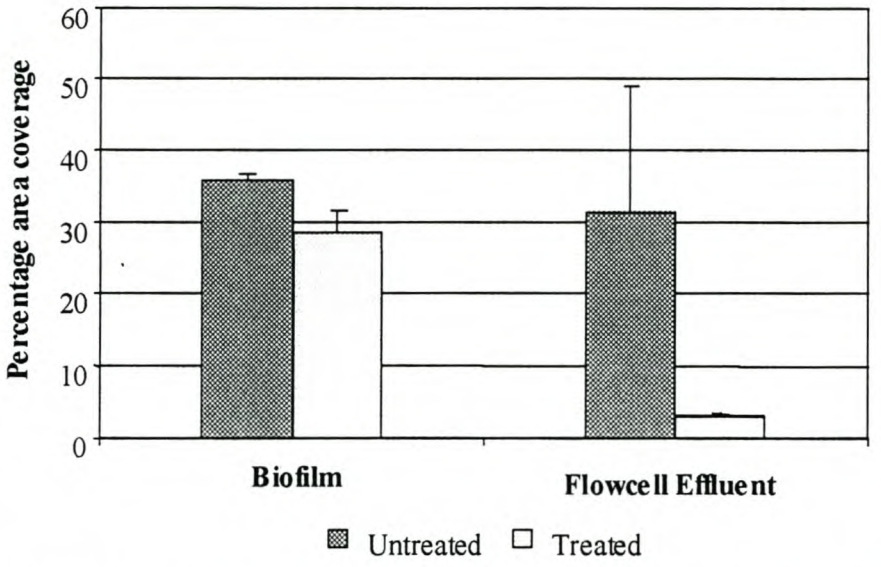


FIGURE 4.7: The percentage area of the attachment surface covered by the biofilm, as well as that of planktonic cells immobilised on filter surfaces, was established before and after biocide treatment. Bacteria from the flowcell effluent were stained with *Bac*Light™ and a standardized volume immobilised on filters to determine the reduction in area coverage after biocide treatment, using image analysis. The difference in percentage reduction in area coverage between the attached and flowcell effluent cells were found to be significant (P = 0.05) ANOVA (STATISTICA, Version 6.0, StatSoft).

When comparing the difference in the decline in the biomass area coverage between the biofilm and flowcell effluent, it was found to be statistically significant (95% confidence interval). The significance of this observation is related to the origin of the planktonic bacteria in the flowcell effluent in Section 4.4.1.3.

4.4.1.3 Determining the origin of the planktonic bacteria in the flowcell effluent.

Once the specific growth rate of isolate CT07 in suspended culture had been determined (FIGURE 4.3), the question regarding the origin of the bacteria in the flowcell effluent could be addressed. According to chemostat theory, the maximum flow rate (F_{max}) at which planktonic dividing cells can be maintained without being washed out of the system, can be calculated by multiplying the dilution rate (D, equal to the specific growth rate, μ) with the

volume of the vessel (V). The internal volume of the flowcell is approximately 200 µl, while the specific growth rate (µ) was calculated as $0.62h^{-1}$ (Section 4.4.1.1).

Therefore:

$F_{max} = 0.62h^{-1} \times 0.2\ ml = 0.13\ ml.h^{-1}$.

Thus, at a flow rate greater than $0.13\ ml.h^{-1}$ in the flowcell, bacteria should not be able to replicate fast enough to prevent the planktonic cells from being washed out.

The applied flow rate was, however, $14.5\ ml.h^{-1}$. The true dilution rate within the flowcell was thus:

$D = F / V = 14.5\ ml.h^{-1} / 0.2\ ml = 72.5h^{-1}$

This is substantially higher than the specific growth rate for planktonic dividing cells ($0.62h^{-1}$). This implies that a planktonic population could not be maintained within the flowcell under these experimental conditions. According to these calculations, it can be hypothesized that, in this instance, the bacterial cells in the effluent from the flow chambers originated from the biofilm.

Daughter cells are thought to be continually released from the biofilm population when it exists in a stress-free environment. The ability of daughter cells to leave the attached community has been considered a mechanism whereby the continued survival of the species is advanced. The daughter cells are assumed capable of leaving one site of colonization, only to search for uncolonized territory where they can establish a confluent biofilm of their own.

This conclusion provides an indirect indication of the physiological state of the biofilm. Approximately 1.55×10^8 CFU/ml were cultured from the flowcell effluent, and if it is assumed that these cells originate from the biofilm based on the above conclusion, then some region of the biofilm must be highly metabolically active.

We hypothesize that an active layer of rapidly dividing cells is located at the biofilm surface; at the interface between the biofilm and the bulk-liquid phase. This has been documented previously in an investigation into the spatial distribution of specific growth rates within *Klebsiella pneumoniae* biofilms, where acridine orange was used to show that the outer 10 to 30 µm layer of cells was more active than those in the interior of the biofilm (Wentland *et al.*, 1999). A higher ratio of RNA to DNA content, which correlates to a high specific growth

rate, was visualised in the layer closest to the biofilm bulk-liquid interface and this was attributed to greater access to nutrients and oxygen.

An explanation for the observations made by Wentland *et al.* (1999) could be provided by the following work. Siebel and Characklis (1991) investigated biofilm formation of *P. aeruginosa* and *K. pneumonia* biofilms as single species, and in combination. They were not only interested in the rate of biofilm formation and biofilm thickness, but also the rate at which the populations consumed glucose and produced specific metabolites. Even though *K. pneumonia* exhibited a maximum specific growth rate five times greater than that of *P. aeruginosa*, the authors determined that both species were present in significant amounts in the binary biofilm, based on biofilm morphology. This contradictory finding was attributed to the greater resistance of substrate diffusion through the thicker (60 μm) and more heterogeneous *K. pneumonia* biofilm, compared to that of the 30 μm thick *P. aeruginosa* biofilm. While the average specific growth rate of the entire *P. aeruginosa* biofilm is determined by the available substrate concentration in the bulk liquid, only the growth rate of the outer layer of cells in the *K. pneumonia* biofilm is regulated in a similar manner. The growth rate of cells in the deeper layers of *K. pneumonia* biofilms is reduced due to limited diffusion of nutrients from the bulk-liquid phase.

4.5.1 Measuring the ability of isolate CT07 to regrow after biocide treatment using the Most Probable Number (MPN) technique.

In order to further investigate the apparent disparity between the percentage of viable bacteria and the CFU/ml after biocide dosage (FIGURES 4.4 and 4.6), the ability of treated planktonic cells to regrow in suspension was tested. Treatment with the biocide may result in injury (bacteriostatic action) to some bacteria, rather than death (bacteriocidal action). The ability of injured bacteria to divide and form visible colonies on plates may be temporarily inhibited, but given time, some of the injured cells may recover to re-colonize the system. Therefore, a liquid-based enumeration system was also evaluated.

Three different concentrations of TSB were employed, namely a 0.3 $g.l^{-1}$, 3 $g.l^{-1}$and 30 $g.l^{-1}$. This was done in order to provide the biocide treated bacteria with a broader concentration range of nutrient conditions, since the recovery ability of injured cells may be enhanced by incubation with additional nutrients.

No regrowth was observed for any of the dilution tubes for the treated samples, thus verifying the observations made on agar plates that the cells lost culturability, despite the maintenance of viability as indicated by the *Bac*Light™ viability probes. Even after prolonged incubation in the absence of the antimicrobial, no regrowth was visible. It may be possible that different results could be obtained with different growth media. However, this was beyond the scope of this study to add more variables.

4.5.2 Determining whether there is a correlation between culturability and viability of isolate CT07 biofilms after biocide treatment.

In order to further investigate the observed difference in the percentage viable cells (as ascertained with the *Bac*Light™ viability probes) and the culturable amount of bacteria following antimicrobial treatment of planktonic populations of CT07, it was decided to subject biofilm cells to similar treatment. The reliability of the *Bac*Light™ viability probes has been questioned before, although observed differences in the amount of viable and culturable bacteria could be attributed to the VBNC phenotype.

The percentage viability of removed and resuspended biofilm bacteria, as determined with *Bac*Light™ viability probe was 24 %, after a 5 hour treatment with the commercial biocide. This corresponded well with the 27 % viability of the intact biofilm. The morphology of biofilm development on the glass slides in the Pedersen device was significantly different from that observed in the flowcells. Biofilm formation on the glass slides was limited to a few clusters, spread across the entire surface, whereas a multi-layered, complex biofilm structure was visible on the glass attachment surface of the flowcells. The difference could be attributed towards the much larger internal volume of the Pedersen device, different flow rates and the recirculation of growth media. The amount of culturable cells from resuspended biofilm bacteria was reduced from 5.1×10^6 to 3×10^4 by treatment with the biocide. This represents a killing efficiency of 99 %, even though the *Bac*Light™ data suggests that 24 to 27 % of the biofilm remained viable after treatment.

As mentioned in section 4.4.1.2, the disparity between the number of culturable cells and the percentage viable cells after biocide treatment was a concern. The number of culturable planktonic cells after biocide treatment was shown to dependent on the rate of growth (Section 4.7), with cells in stationary phase showing the greatest ability to survive. While no cells could be cultured from the faster growing planktonic bacteria, the physiological probes indicated that some cells retained viability after antimicrobial treatment. These cells could

simply be injured as a result of the treatment, and were in the process of dying, since no regrowth of treated planktonic bacteria was observed.

It should be pointed out that culturability, expressed as CFU/ml, provides the ability to detect log-fold changes in cell numbers. Percentage viability determinations, however, can only range from 0 to 100. A reduction in cell numbers from 1.2 x 10^9 to 6.6 x 10^6 CFU/ml upon antimicrobial treatment (FIGURE 4.6), represent a 3-fold log reduction, but corresponds to a 99 % killing efficiency. The surviving 6.6 x 10^6 CFU of bacteria per millilitre still poses a significant problem in terms of the control of biofouling in industrial systems.

4.6.1 Measuring protease activity of the isolates CT01 to CT07.

The reliability of the various techniques to estimate cellular viability, using fluorescent probes, has been questioned and alternatives, such as the possible difference in protease activity between viable and compromised bacteria, were explored. Only isolates CT01 and CT07 displayed protease activity as indicated by clearance zones on the Luria Bertani Broth Skim Milk plates after incubation for 2 to 3 days at 30°C.

The determination of protease activity in liquid culture with the modified method of Long *et al.* 1981 as described by Deane *et. al.* (1987) only showed a significant detectable amount of protease activity for isolate CT01 after 48 hours (TABLE 4.2).

TABLE 4.2: The seven cooling tower water isolates were screened for the production of proteases after 24 and 48 hours at an optical density of 440nm. One unit of protease activity is defined as the amount of enzyme that gives an increase in absorbance of 0.1 at 440 nm in 30 minutes at 37°C.

	Incubation time	
Isolate	**24 Hours**	**48 Hours**
CT01	0.019	0.240
CT02	0.005	0
CT03	0	0
CT04	0	0.002
CT05	0	0
CT06	0	0
CT07	0	0.024

4.6.2 Determining the protease activity of flowcell effluent.

The possibility of using protease activity as a measure of viability in a fluid system, such as that of a flowcell, does not seem to be a viable option. As is evident from the results summarised in TABLE 4.3, very little protease activity was detected in the flowcell effluent before treatment and as such, no meaningful comparison could be made. It is possible that the proteases were immobilized in the EPS matrix with little release into the bulk solution.

TABLE 4.3: Flowcell effluent from untreated and biocide treated flow chambers of different ages, were collected and assayed for the production of proteases at an optical density of 440nm.

	Incubation time		
	Day4	**Day7**	**Day14**
Untreated effluent	0.016	0	0.004
Treated effluent	0	0	0

4.7 The antimicrobial resistance of batch cultures of isolate CT07 in the three different growth phases.

Isolate CT07 was able to survive in the presence of the biocide when cultured as a planktonic culture in a chemostat as was shown in Section 4.4.1.2. Previous work done by Spoering and Lewis (2001) showed that *P. aeruginosa* biofilms, and planktonic cells in the stationary phase of growth have similar abilities to resist killing by antimicrobials. Indeed, in their examination of the effects of four antimicrobials with diverse modes of action, they found that stationary-phase cells were moderately more resistant to killing than biofilm bacteria. They attributed this ability to survive antimicrobial treatment to slow growth and the presence of persister cells – a small fraction of cells seemingly impervious to killing.

This observation prompted the investigation into the ability of planktonic cultures of isolate CT07 to survive the presence of a biocide, when in different growth phases.

The number of culturable colonies from the different growth phases, before biocide dosage increased from 4.9×10^7 CFU/ml in the lag phase to 2.3×10^9 CFU/ml in the stationary phase (FIGURE 4.8).

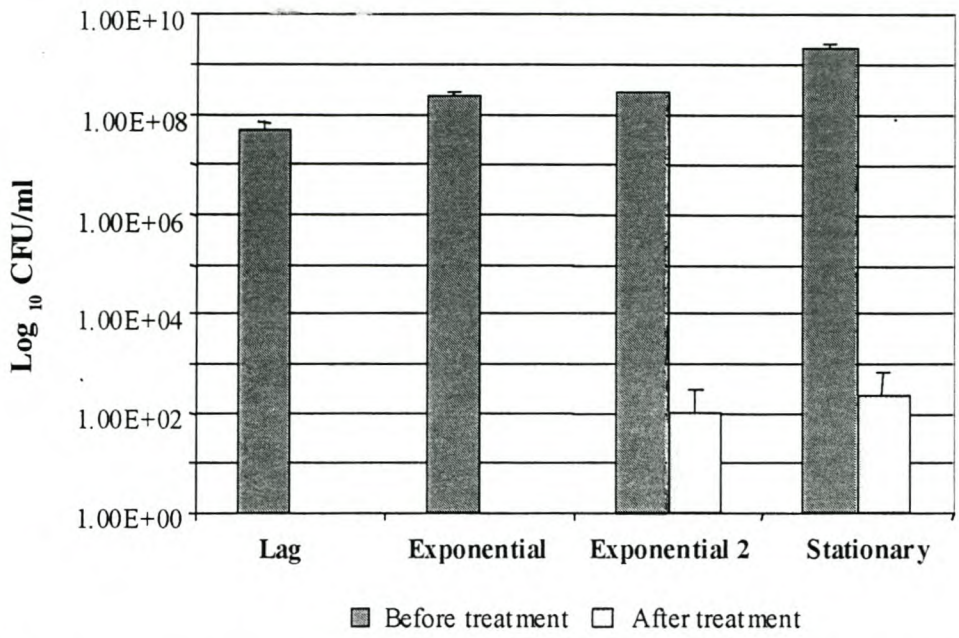


FIGURE 4.8: Colony counts were performed on samples taken from the batch cultures, in the different growth phases, prior to and after biocide treatment. The bacteria were cultivated on 3 $g.l^{-1}$ TSA plates and incubated at room temperature for 2 – 3 days. The experiment was performed in triplicate.

No colonies could be cultured from the lag and first exponential phase bacteria after incubation with the biocide. This was contrasted by the presence of growth of biocide treated cells from the second exponential and stationary phases. Approximately 1.1 x 10^2 and 2.3 x 10^2 CFU/ml were cultured from the treated second exponential phase and stationary phase, respectively. This trend correlates with the viability results as determined with *Bac*Light™ (FIGURE 4.9). It should, however, be pointed out that the amount of culturable cells decreased by several logs, and this corresponds to a near 100% killing efficiency.

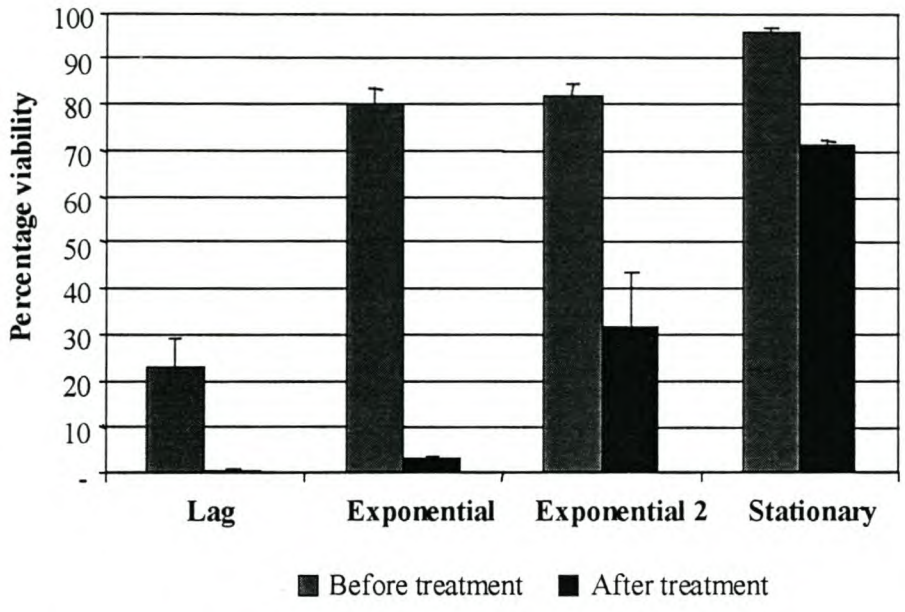


FIGURE 4.9: Batch cultures of isolate CT07 in different growth phases were treated with the commercial biocide and cellular viability was determined using *Bac*Light™ viability probes. A significantly different percentage of cells, taken from each of the growth phases survived antimicrobial treatment, except when killing of the lag phase cells was compared to that achieved in the first exponential phase (P = 0.05) (ANOVA, STATISTICA, Version 6.0, StatSoft). The efficacy of the biocide decreased significantly as the bacterial cells progressed from lag phase to the exponential phase and reached the stationary phase. The experiment was performed in triplicate.

These results indicate that the majority of the chemostat-derived planktonic cells were in the stationary phase of growth, which allowed for the survival of a part of the original population after challenge with an antimicrobial agent (FIGURE 4.4 and 4.6).

From these results, it could be concluded that the ability of isolate CT07 to resist treatment with a biocide is positively related to growth rate. Actively growing and dividing bacteria, as those in the exponential phase of growth, are more susceptible to antimicrobial treatment than cells in the stationary growth phase.

The results presented here, fits well with the hypothesis of an active layer of cells at the biofilm bulk-fluid interface. A rapidly dividing, metabolically active layer of the biofilm would be more sensitive to treatment with an antimicrobial, resulting in killing and/or injury. The result of this is the cessation of cell division, and consequently, a decline in the number of cells shed from the biofilm. Not only did the viability and culturability of the cells shed

from the biofilm decrease significantly in response to antimicrobial challenge (FIGURES 4.4 and 4.6), but also the total number of daughter cells shed from the biofilm (FIGURE 4.7).

The majority of cells within the deeper layers of the biofilm are likely to be slow growing. This could be partially due to limited diffusion of oxygen and nutrients through the EPS enclosed matrix of cells. These bacteria would be less sensitive to biocide addition, and better able to survive, especially if the uppermost layer of previously active cells is removed from the biofilm by the biocide, since oxygen and nutrient diffusion would then be less restricted.

The presence of such relatively inactive cells within the biofilm could explain the insignificant loss of biofilm viability and biomass after biocide addition, while the amount of cells shed from the biofilm decreased significantly as a result of the inhibitory action of the antimicrobial on the metabolically active layer of the biofilm. This scenario would be even more likely if highly reactive biocides, such as glutaraldehyde or chlorine are applied. The outer layers of the biofilm may be sacrificed to quench the activity of the agent, thereby providing protection to the remaining cells.

4.8 The biofilm growth rate of isolate CT07.

In order to compare the growth rate of isolate CT07 in batch culture (i.e. planktonic) to the growth rate of a biofilm at a surface, we observed the accumulation of bacteria at a surface during the early stages of biofilm development (FIGURE 4.10 and FIGURE 4.11). Only counts of the number of cells at the glass attachment surface (*xy* plane) were taken and biofilm development in the *xz* plane was disregarded. Three dimensional microcolony formation was observed after approximately 13 hours.

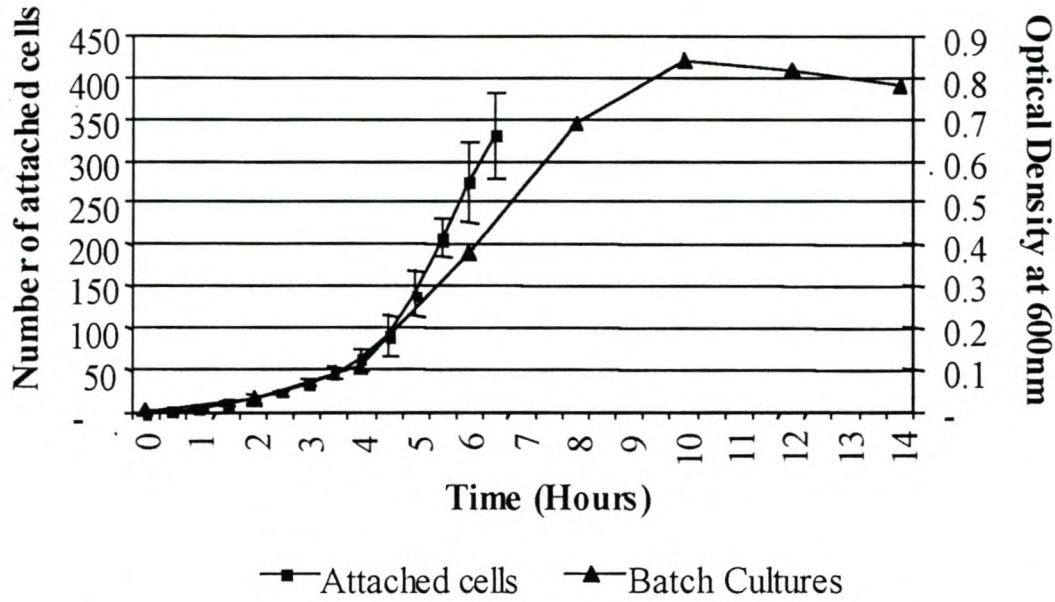


FIGURE 4.10: The growth rate of isolate CT07 in a 3 $g.l^{-1}$ TSB batch culture at room temperature was compared with the rate of attachment and growth on a glass surface over time. Growth in batch culture was quantified according to the increase in optical density, while the number of cells present at the flowcell surface was counted to determine the biofilm growth rate during the early stages of biofilm formation. The flowcell was inoculated with 1 ml of an overnight culture, with the media flow stopped for 30 minutes. The flow was resumed at 14.5 $ml.h^{-1}$ for 12 hours, before the accumulation at the glass surface was quantified at 30-minute intervals (time 0 and onward on the x-axis).

The specific growth rate of isolate CT07 in batch culture was calculated as 0.62 h^{-1}, in Section 4.4.1.1, while the specific growth rate (μ) as calculated from the attached cell counts at the glass surface of the flowcell is 1.2 h^{-1}.

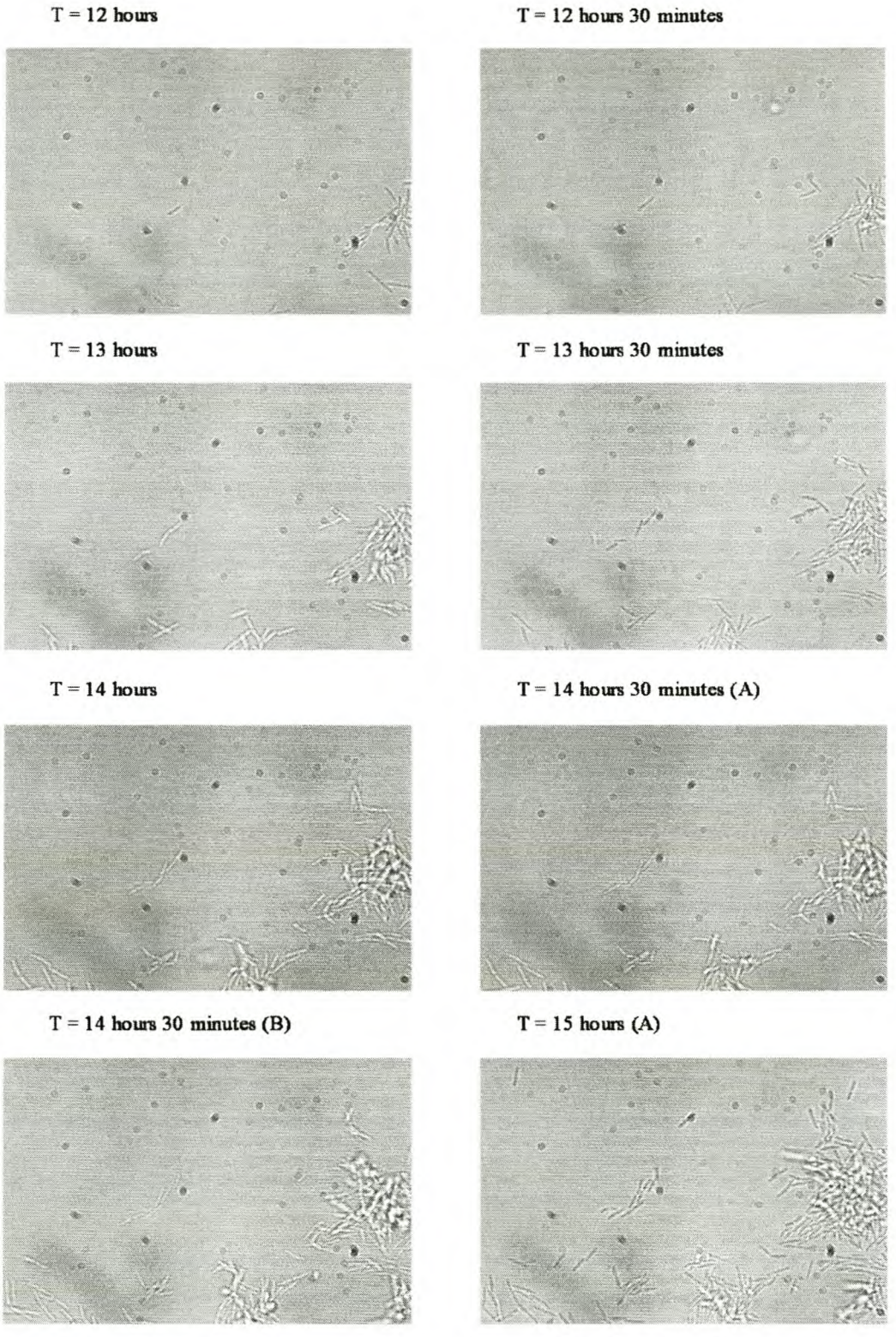
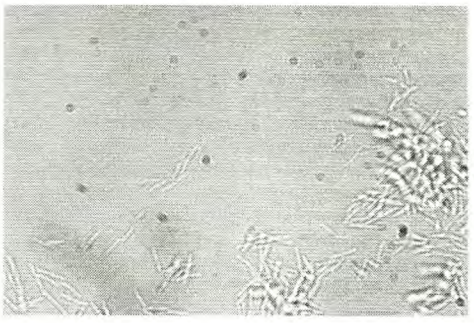
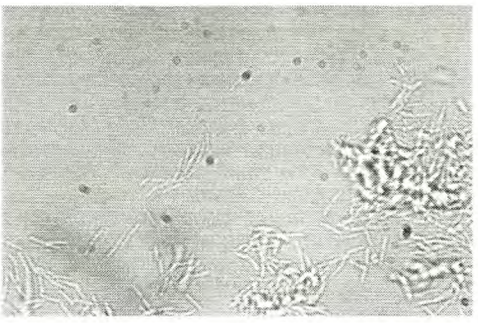


FIGURE 4.11: Light microscopy images taken of biofilm formation, at the same location, of isolate CT07 over a period of 20 hours. The biofilm was cultivated in a flowcell, on 3 $g.l^{-1}$ TSB at a flow rate of 14.5$ml.h^{-1}$, at room temperature.

T = 15 hours (B)



T = 15 hours 30 minutes (A)



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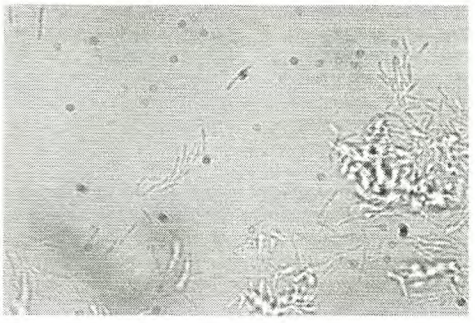

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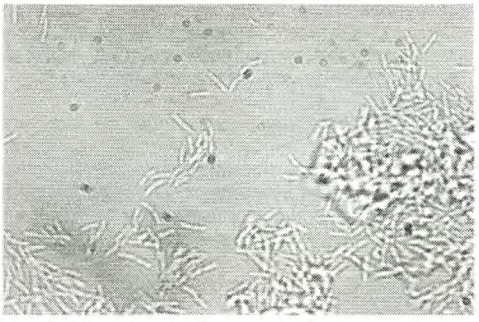

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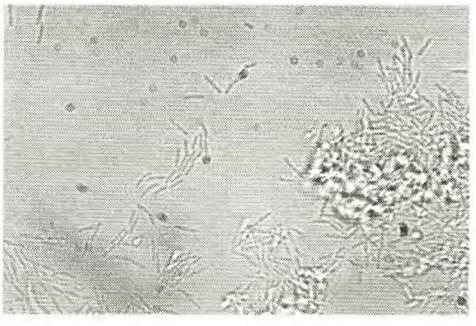
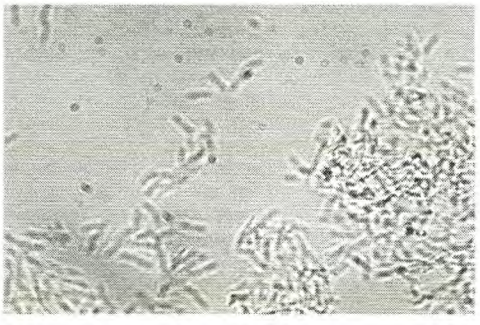


T = 16 hours (C)



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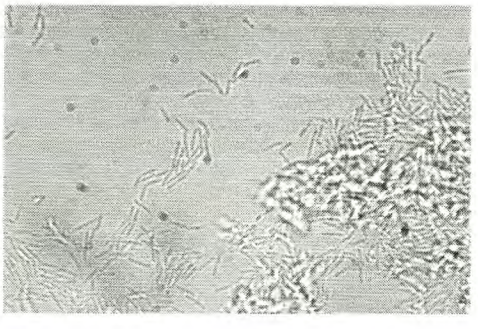

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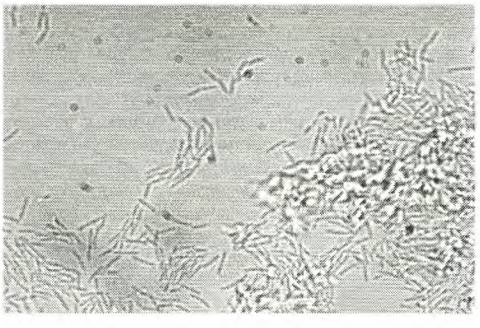

FIGURE 4.11: Continued (2).

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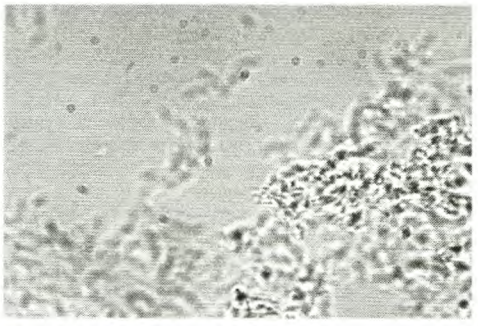

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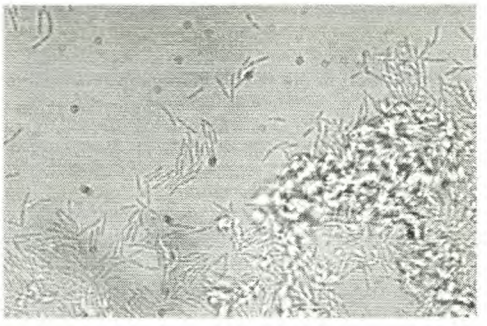

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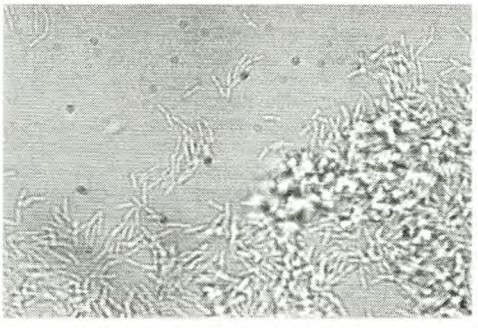

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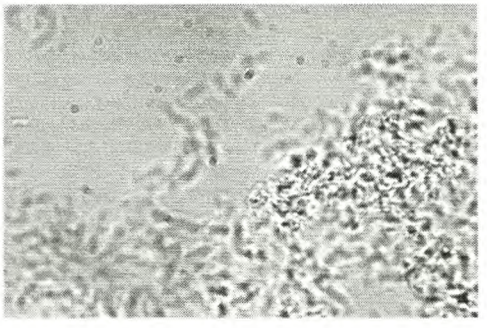

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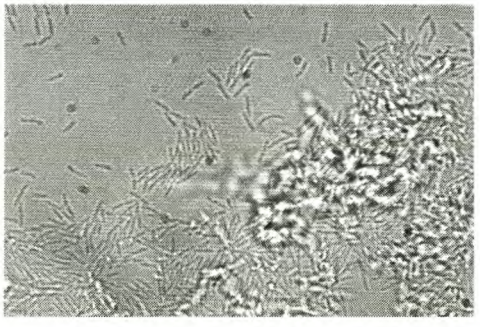

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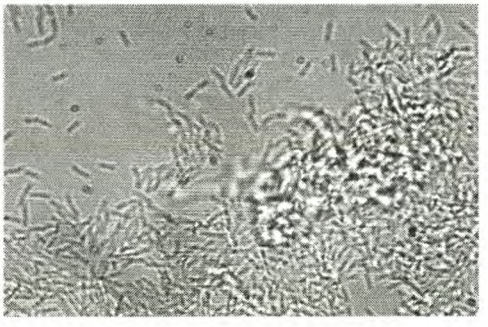

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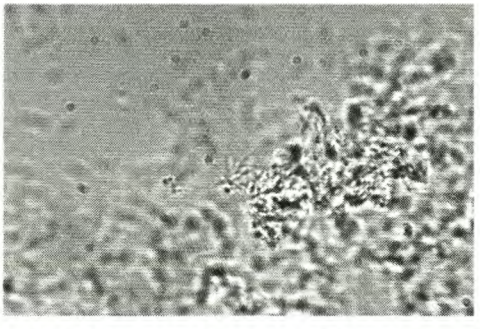

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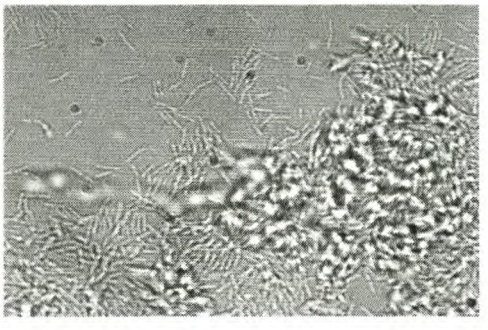

FIGURE 4.11: Continued (3).

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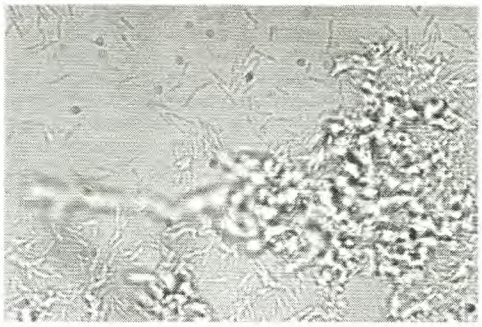

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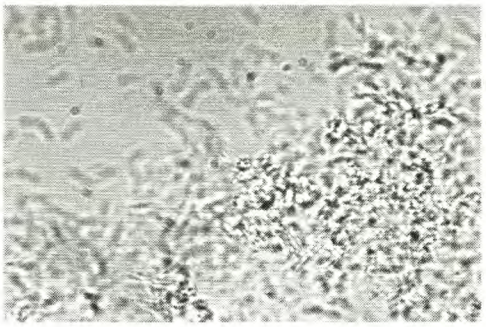

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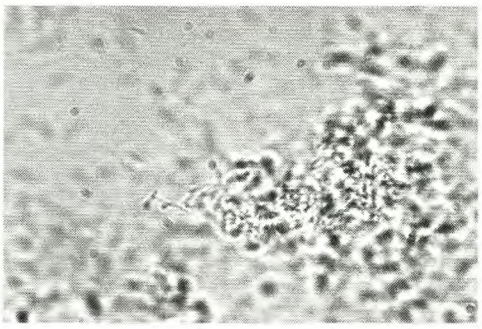

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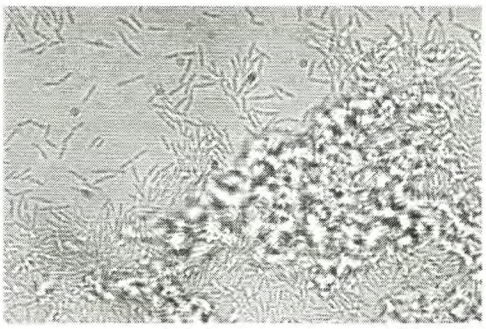

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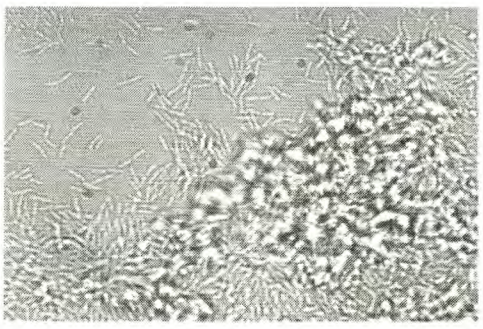

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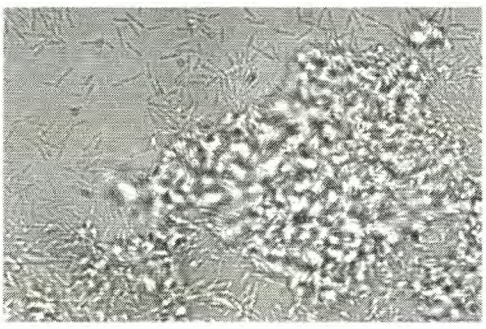

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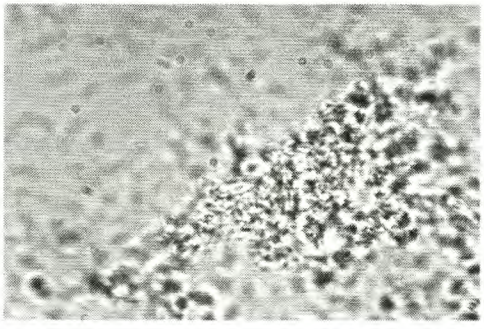

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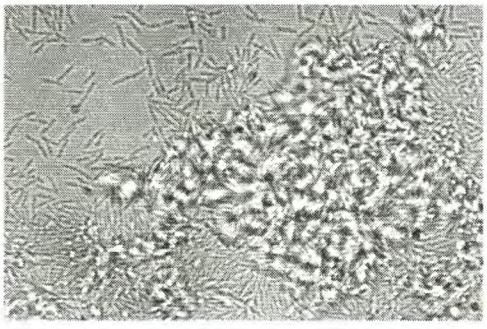

FIGURE 4.11: Continued (4).

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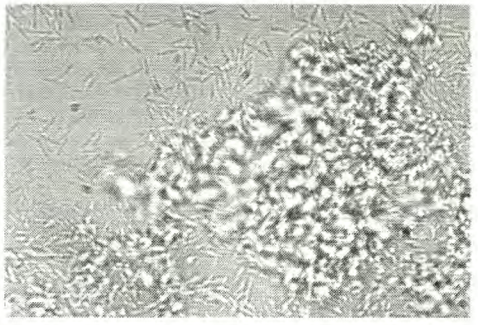

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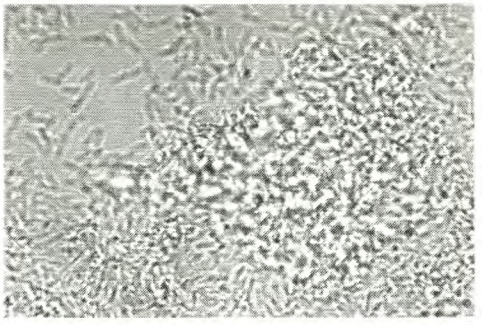

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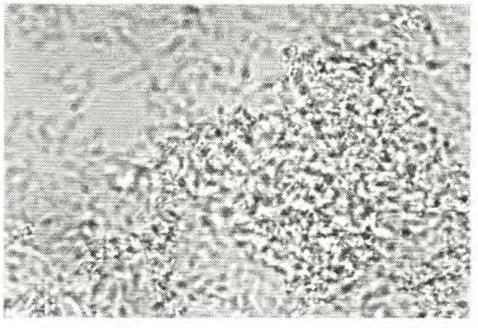

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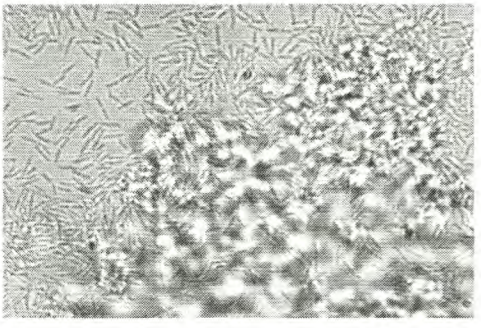

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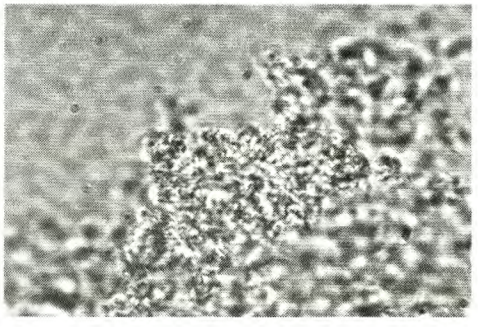
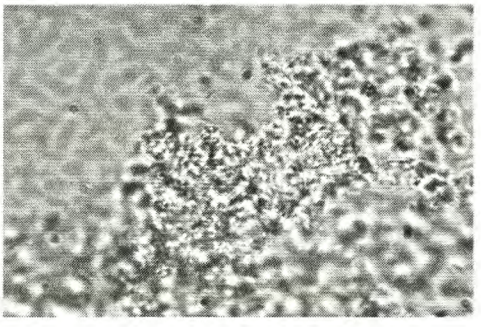


T = 19 hours 30 minutes (C)



T = 19 hours 30 minutes (D)

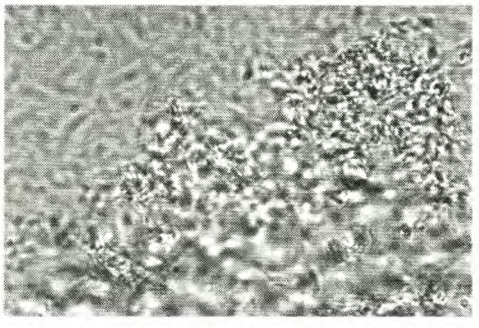


FIGURE 4.11: Continued (5).

Another approach was used to quantify the accumulation of isolate CT07 at a surface. An Optical Large Area Photometer (OLAPH) detected the accumulation of cells at the plexiglass surface under conditions of continuous flow using 3 $g.l^{-1}$ TSB as growth media. The advantage provided by this approach was in the detection of the accumulation of cells beyond a monolayer. Whereas the previous approach, involving microscopy, allowed for a direct quantification of cells, the photometer detected three dimensional biofilm formation and provided for indirect quantification of this process over a longer period of time (FIGURE 4.12).

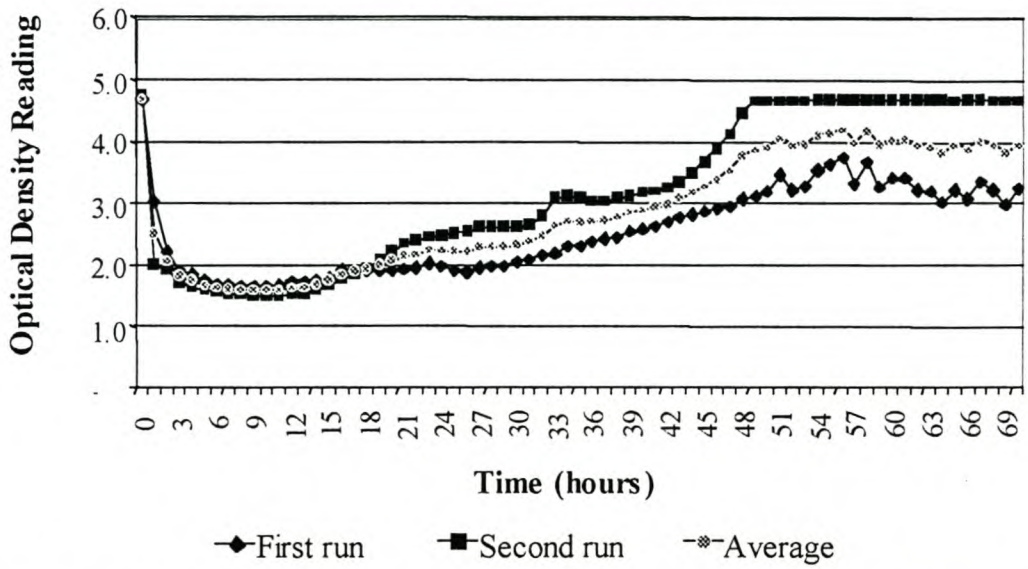


FIGURE 4.12: The attachment of isolate CT07 at a plexiglass surface was determined with an Optical Large Area Photometer (OLAPH) over a period of 70 hours at room temperature. This was duplicated in order to calculate an average growth curve.

An initial decrease in biomass in the flowcell was recorded as the unattached inoculum washed out after flow was resumed. This was followed by a lag phase of approximately 15 hours, and a rapid increase in the number of attached cells over the ensuing 33 hours. The biofilm reached its maximum thickness under these growth conditions soon thereafter. This approach, based on optical density, provided evidence that the initial establishment and subsequent biofilm development spanned 48 hours. This was followed by a relatively stable maintenance of biofilm thickness, with small variation, presumably due to detachment of parts of the biofilm and regrowth to the original thickness.

As evident in FIGURE 4.12, there was a marked difference in the optical density profile between the different experiments. This can be attributed to spatial variability of the attachment surface between experiments. It may, however, also indicate the variability in behaviour of microbes at surfaces, even when grown in pure culture. Lewandowski *et al.* (2003) recently reported on their efforts to generate structurally reproducible biofilms. Despite rigorous efforts to follow precise methodology, the cultivation of structurally comparable biofilms over long periods was not successful. They observed that duplicate biofilms only exhibited similar structures until the first sloughing of the biofilm occurred, where after the pattern of biofilm formation varied.

When growing in a continuous culture, such as a chemostat or flow chamber, the growth rate of the free-floating bacteria in the bulk-liquid phase may differ from that of the bacteria attached to the surface. Biofilm growth at the surface is not dependent on the maximum dilution rate, as reported by Larsen and Dimmick (1964). When the dilution rate of a chemostat system was increased above that of the maximum specific growth rate of *Serratia marcescens*, no complete washout of cells occurred. This was attributed to the detachment of cells from the surface of the chemostat. Van Loosdrecht *et al.* (1990) commented that large numbers of surface attached bacteria may act as a buffer system, preventing the complete loss of microbial biomass from an environment when dilution rates were altered or perhaps when challenged with an antimicrobial, as was the case in this study.

Van Loosdrecht *et al.* (1990) commented on an experiment performed by Ellwood *et al.* (1982) wherein it was observed that a *Pseudomonas* sp. accumulated at a glass surface in continuous-culture, at a dilution rate of 0.06 h^{-1}. This rate indicated that the initial increase of cells at the surface occurred almost twice as rapidly than that of growth in suspension. They assumed that the contribution of cells from the suspended phase to the surface-associated population was negligible and concluded that the greater rate of increased growth was mainly the result of growth at the surface.

Isolate CT07 biofilms exhibited a high initial growth rate, with microcolony development in the third dimension within 13 hours of inoculation with a stationary overnight culture (FIGURE 4.11). The formation of an extensive multi-layered architecture suggests that the outer regions of the biofilm are optimally positioned to benefit from easy access to nutrients and oxygen from the bulk fluid. In fact, if the assumption is correct that the planktonic population cannot persist at these dilution rates that far exceed the maximum specific growth rate of the organism, then there should be a zone in the biofilm with high cell turnover.

Based on observations that cells within developed microcolonies remain in place over long periods of time, it is most probable that the outer regions of the biofilm is primarily responsible for the cells present in the effluent. The fact that the biofilm biomass remained constant after 45-50 hours (FIGURE 4.12) while the effluent cell numbers remained high, suggests high growth rates at the biofilm-liquid interface. It is possible that growth rates similar to that observed during initial biofilm formation (FIGURE 4.11) are maintained in this region.

The increase in biofilm surface-to-volume ratio, by the development of an uneven surface area, increases the amount if active cells at the biofilm-liquid interface and may account for the continued shedding of high numbers of daughter cells into the effluent, to the degree that this region may be viewed as a "cell-nursery".

A visual representation of the hypothesis proposed in Section 4.4.1.3 (that an active layer of rapidly dividing cells is located at the outer surface of the biofilm) is provided in FIGURE 4.13).

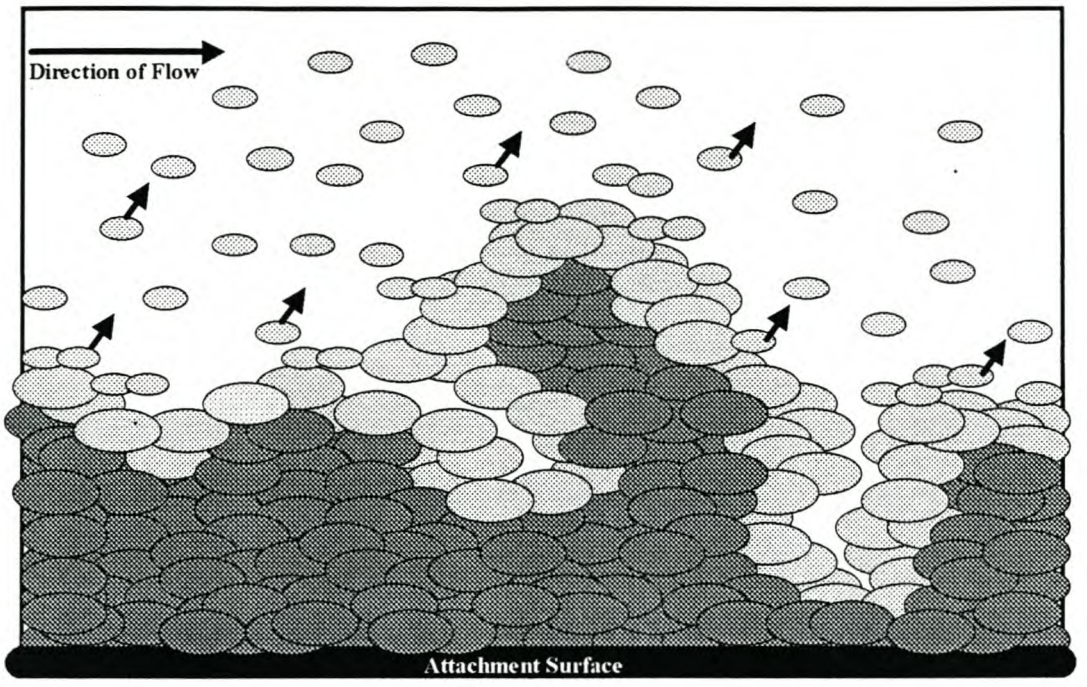


FIGURE 4.13: A diagrammatical representation of the hypothesis regarding the distribution of metabolic activity in a CT07 biofilm. The light cells have better access to nutrients and oxygen from the bulk-liquid phase and are able to maintain a high metabolic activity and division rate. A large number of daughter cells are shed from this active layer. The darker cells are nutrient and oxygen limited, due to retarded diffusion within the biofilm matrix. Division of these bacteria are also likely to be limited due to space constraints and the presence of EPS.

Recent work by Walters *et al.* (2003) corresponds well with the results presented here. They showed that oxygen limitation and lowered metabolic activity did influence the ability of *P. aeruginosa* colony biofilms, cultivated on filters placed on agar plates, to tolerate ciprofloxacin and tobramycin. Their work confirmed that although both antibiotics diffused through the entire biofilm, it did not result in a substantial decline of culturable bacteria similar to those of antibiotic-treated planktonic cells (i.e. 5.2 ± 0.2 and 5.9 ± 0.1 log reductions for ciprofloxacin and tobramycin treated planktonic bacteria, compared to a 1.42 ± 0.03 and 0.49 ± 0.18 log reduction, respectively, for colony biofilm bacteria after a prolonged exposure time of 100 hours).

Transmission electron microscopy was utilised to confirm that antibiotic action was only evident in bacteria near the air-biofilm interface. Visual evidence of antibiotic action included bloated and vacuolated cells, cell fragments, lysed cells, filamentation and cell

ghosts. Microelectrode measurements verified that oxygen penetrated only 50 to 90 μm into the 233 ± 26 μm thick biofilm. This region corresponded to the metabolically active zone, as visualised by the induced expression of green fluorescent protein (GFP) (Walters *et al.*, 2003).

Measurements of average specific growth rate of the biofilm colonies over time corresponded to the evidence of metabolic activity gradients as implied by protein synthesis (GFP induction). While rapid bacterial accumulation was observed within the first 6 hours of colony development (specific growth rate of $0.66 \pm 0.04 h^{-1}$), this declined to $0.013 \pm 0.009\ h^{-1}$ during the last 24 hours of development. Prior to antibiotic treatment, the average specific growth rate was only approximately 2 % of the initial tempo, which the authors suggested pointed to replication only at the edges of the colony biofilm (Walters *et al.*, 2003).

The use of confocal laser scanning microscopy (CLSM) in conjunction with *Bac*Light™ viability probes have been used to indicate that microcolonies of oral biofilms, cultivated in vitro, can have defined outer layers of viable bacteria and inner cores of non-viable cells (Hope and Wilson, 2003). This was not, however, a consistent observation, as layers of non-viable bacteria was also present at the bulk-liquid interface of the biofilm. The possibility that the distinct layers of viable and non-viable bacteria can be attributed to differential penetration of the two *Bac*Light™ components into the biofilm was discounted since the components have comparable sizes (Component A = 550-750 Da and Component B = 668.4 Da) and positive charges.

Evans *et al.* (1991) compared the susceptibility of *P. aeruginosa* and *E. coli* biofilms in different growth rates to ciprofloxacin. A mucoid (PaWH) and non-mucoid strain (PaTM) of *P. aeruginosa* were cultivated in chemostats at different specific growth rates. A membrane-based cultivation method was used to grow biofilms to various steady-state growth rates, with continuous irrigation of growth media. The susceptibility of the both chemostat cultures increased in proportion with the specific growth rate. The slow growing non-mucoid biofilms seemed to be more sensitive to ciprofloxacin (0.5 mg/l), while fast growing PaTM biofilms were significantly more resistant than planktonic cultures. The PaWH biofilms were hardly affected by ciprofloxacin. Thereafter, a 10 times higher dosage of ciprofloxacin (5 mg/l) was used against the mucoid strain, and it was shown that susceptibility increased with a faster growth rate for both the chemostat and biofilm cultures, although the biofilm still remained significantly more resistant. The daughter cells that eluted from the biofilm cultures showed a significantly greater susceptibility to ciprofloxacin than any of the other cultures, and showed no correlation with the specific growth rate of the parent biofilm cultures.

The results for chemostat cultures of *E. coli* was similar to that of *P. aeruginosa* in that it showed significant increases in susceptibility to ciprofloxacin with an increase in growth rate. Conversely, the cells resuspended from the colony biofilms, cultivated on filters, did not indicate a decrease in resistance. Cells shed from the biofilm also showed a greater sensitivity towards the antibiotic. The intact biofilm did not have a significantly greater resistance to ciprofloxacin than suspended cells from the chemostat. The relative resistance of mucoid *P. aeruginosa* biofilms to the elevated concentration of 5 mg/l ciprofloxacin supports the suggestion that the structural organization of these cells within an extrapolymeric matrix confers some form of resistance (Evans *et al.* 1991).

4.9 General Conclusions

Initial investigation into the ability of biofilm and planktonic populations in an operational cooling tower to survive treatment with a commercial biocide was inconclusive. Subsequent laboratory experiments involved a bacterial community, isolated from cooling tower water. Attached populations, as well as those in the flowcell effluent, were subjected to treatment with an antimicrobial, but no significant difference in antimicrobial susceptibility was observed.

This led to speculation of the origin of the planktonic cells in the effluent from the flowcells. The specific growth rate of one of the isolates from the cooling tower water, CT07, was determined. Calculations indicated that multiplication by the planktonic population alone would be unable to overcome the growth medium dilution rate present in the flowcells, resulting in washout from the system. This indicated that the bacterial cells in the flowcell effluent originated from the biofilm.

Upon comparing the susceptibility of attached, planktonic (chemostat-grown) and biofilm-detached bacteria to treatment with the antimicrobial agent, a significant difference in percentage viability between attached and detached biofilm cells was observed ($P = 0.05$). No significant difference in antimicrobial resistance was detected between planktonic and attached populations, although the percentage viability of biofilm bacteria decreased less than that of the chemostat-derived planktonic cells.

Antimicrobial resistance was shown to be dependent on the growth rate of the bacterial populations, with an increased ability to survive associated with a decrease in specific growth rate. This led to the hypothesis that a metabolically active layer of cells is present at the bulk-liquid interface in biofilms. These cells grow, divide and shed high numbers of daughter cells into the effluent (~1.55×10^8 CFU/ml). Treatment with an antimicrobial agent resulted in a significant reduction in the viability and number of cells shed from the biofilm, indicating that the metabolically active layer of the biofilm was inhibited by treatment, and by inference exhibited a higher specific growth rate. The bacterial cells in the layers closer to the attachment surface of the biofilm has frequently been shown to be slow growing, due to nutrient and oxygen limitation. Furthermore, the inhibition of the active layer of the biofilm by antimicrobial treatment could protect the deeper lying cells from harm. It is possible that an important function of this deeper layer is indeed to ensure the continued survival of the population under adverse conditions. Thus there is an interesting diversification; proliferation by the outer layers under favourable conditions, and survival of the deeper layers during less favourable environmental circumstances.

Growth of the bacteria at the surface revealed an initial high specific growth rate, exceeding that of a planktonic population of the same strain. Accumulation of biomass at the surface, measured by optical density changes also indicated an exponential stage, and subsequently a gradual deceleration until the biofilm reached a fully developed state after approximately 48 hours. Fluctuation of the amount of attached biomass following the initial period presumably indicates the detachment of parts of the biofilm, and regrowth, or attachment of bacteria from the planktonic phase. This supports the hypothesis that the surface of the biofilm remains metabolically active and capable of responding to environmental stresses, such as the presence of an antimicrobial agent.

The results presented here, support hypotheses put forth in literature to account for the increased resistance of biofilms to the negative effects of antimicrobial agents. Future work will include an investigation into changes in the patterns of gene expression when a bacterium becomes attached to a surface, as well as upon release from the biofilm, and the influence this has on the ability to resist antimicrobial treatment.

APPENDIX

Azocasein solution:

1.21 g Tris
2.34 g NaCl
0.029 g $CaCl_2.2H_2O$
Make up with distilled H_2O to 100 ml.
Autoclave.

Add 2 grams azocasein fibres and stir overnight.
Store at 4°C.

Luria Bertani Broth Skim milk plates:

11.25 g Luria Bertani Broth (Biolab)
10 g Bacteriological Agar (Biolab)
Make up to 375 ml with distilled H_2O.

12.5 g Fat free skim milk powder
Make up to 125 ml with distilled H_2O.
Autoclave the two mixtures separately.
Combine the two mixtures before pouring the plates.

3 $g.l^{-1}$ Tryptone Soy Broth Agar Plates (TSA):

3 g Tryptone Soy Broth (Biolab)
12 g Bacteriological Agar (Biolab)
Make up to 1 L with distilled H_2O.
Autoclave.

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