MICROBIAL RESPONSE TO OXIDISING BIOCIDES

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

Biofouling of water systems is a problem extensively experienced in industry. Although this subject is the focus of many studies, the ability of microorganisms to survive exposure to biocides is still poorly understood. This study aimed to assess the biocidal effect of ozone on planktonic cells and biofilm communities, to evaluate different ozone generation techniques, and to follow population shifts within the biofilm community. Specific objectives included determining the effect of different ozone concentrations, the effect of different exposure times, and an assessment of microbial responses after exposure to sub-lethal ozone concentrations. Typically, 300 ml of an overnight bacterial culture was exposed to ozone that was generated by anodic oxidation (0.3% wt or 18-20% wt, respectively) or silent electric discharge (3.5% wt O_3). The ozone was purged into the culture for 5-, 7-, 10- and 15 min., respectively. Enumeration of cells following ≥10 min. exposure to 18-20% wt ozone showed a significant reduction in viable cell numbers. In contrast, when exposed to the two lower O_3 concentrations, there was little change in the viable cell numbers even after prolonged exposure (30- and 60 min.). To evaluate biofilms, ozone was bubbled into the irrigation that was pumped through replicate flow cell channels. Response to ozone exposure was evaluated after staining the biofilms with the Baclight Viability probe, observation with fluorescence microscopy, and image analysis. The higher ozone concentration (18-20% wt O_3) more effectively disrupted the biofilm structure of denser biofilms than the lower concentration, especially after 90 min. exposure. When compared to the controls, the 90 min. exposure resulted in a notable reduction in viable cells from 69% to 38% and a corresponding increase in non-viable cells from 29% to 62%. The lower concentration ozone (3.5% wt O_3) was effective against the less dense, thinner biofilms evaluated, but not effective against the thicker biofilm. An analysis of the differences between continuous culture biofilms and batch culture biofilms showed that the biofilms in the batch system were less rigid. To evaluate microbial response to biocides, techniques such as Biolog whole-community metabolic profiles and terminal restriction fragment length polymorphisms (T-RFLP) were used. Biolog analysis of planktonic cells revealed changes following exposure to sub-lethal biocide concentrations, however carbon utilisation profiles resembled that of the controls after 24-48 hours. For biofilm communities, no carbon utilization

differences could be detected under these conditions. There was, however differences in T-RFLP patterns between treated and untreated biofilm communities.

OPSOMMING

Biobevuiling van watersisteme is 'n probleem wat algemeen in industriële ervaar word. Alhoewel hierdie onderwerp die fokus van vele studies is, word die vermoëns van mikroorganismes om blootstelling aan biosiede te weerstaan steeds swak verstaan. Die doel van hierdie studie was om die biosidiese effek van osoon op planktoniese selle en biofilm gemeenskappe waar te neem, om die verskillende osoon generasie tegnieke te evalueer, asook om verskuiwings in die samestelling van die biofilm gemeenskap waar te neem. Spesifieke doelwitte sluit in die bepaling van die effek van verskillende osoon konsentrasies, die blootstellingtye, en 'n waarneming van mikrobiese reaksies na blootstelling aan sub-dodings osoon konsentrasies. Drie honderd ml van 'n oornag bakteriese kultuur was aan osoon, wat deur anodiese oksidasie (0.3% wt of 18% - 20% wt) of geluidlose elektriese ontlading (3.5% wt), gegenereer is, blootgestel. Tye van blootstelling was 5-, 7-, 10-, of 15 min., onderskeidelik. Bepaling van selgetalle na ≥10 min. blootstelling aan 18 - 20% wt osoon, het 'n betekenisvolle verlaging in die getal lewensvatbare mikrobeselle getoon. In teenstelling hiermee, het blootstelling aan twee laer osoon konsentrasies min verskil in die lewensvatbare selgetalle, selfs na verlengde blootstellingstye (30- en 60 min.), getoon. Om biofilms te evalueer is osoon in die medium geborrel wat deur replikaat vloeisel kanale gepomp is. Na osoon blootstelling, was die vloeisel onderwerp aan beeld analise deur gebruik te maak van die Baclight lewensvatbare peiler en fluoressensie mikroskopie. Die hoër osoon konsentrasie (18 - 20% wt O_3) het die struktuur van dikker biofilms meer effektief uiteengeskeur as die laer konsentrasie, veral na 90 min. blootstelling. In vergelyking met die onderskeie kontroles, het die getalle van lewensvatbare selle na 90 min. blootstelling drasties verlaag vanaf 69% tot 38% en 'n ooreenstemmende toename in die nie-lewensvatbare selgetalle vanaf 29% tot 62%. Die laer osoon konsentrasie (3.5% wt O_3) was meer effektief teenoor die minder digte en dunner biofilms wat ge-evalueer was, maar nie so effektief teenoor die dikker biofilms nie. 'n Analise van die verskille tussen kontinue-kultuur biofilms en lot-kultuur biofilms het getoon dat die lot-kultuur biofilms minder rigied is. Vir die evaluering van mikrobiese reaksies na biosied blootstelling, is tegnieke soos Biolog gemeenskap metaboliese profiele en eind-restriksie-fragment-lengte polimorfisme (T-RFLP) gebruik. Biolog analise van planktoniese selle het verskille getoon na

blootstelling aan sub-dodelike biosied konsentrasies. Koolstof benutting het wel na 24 - 48 ure met dit van die kontrole ooreengestem. Vir biofilm gemeenskappe was daar geen noemenswaardige verskille in koolstof benutting nie. Daar was wel verskille in T-RFLP patrone tussen die onbehandelde en biosied-behandelde biofilm gemeenskappe.

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CONTENTS

CHAPTER 1. INTRODUCTION & OBJECTIVES.

Water is a life sustaining natural resource and its utilization should be managed, controlled and conserved. There is, therefore, a need to manage natural water supplies such as lakes, rivers and springs, as well as waters used in industry for cooling, washing, dust suppression, etc. in such a way to ensure long-term water supply. Widespread in all these waters are biofilms, which are microorganisms associated with surfaces in almost all water bodies. Biofilm deposition causes significant economical losses (Flemming, 1996). Biofilm production has been studied in various industrial water systems, where chlorine and a number of other biocide types have been used to control biofilm formation. Due to biocidal inefficiency, these water systems often experience biofouling related problems (Hamilton *et al.*, 1970). The cost of heat exchanger fouling in the United Kingdom was quoted by McCoy (1987) to be estimated at over £500 million. Treated water may be subject to conditions in the distribution network that adversely affect it. Bacterial numbers could therefore be increased during distribution by the introduction from external sources, such as open reservoirs, breakages in pipelines, etc. (Rossie, 1975). Another factor for increase in bacterial numbers is the situation where regrowth, or aftergrowth of bacteria occurs (Nagy and Olson, 1985). Biofilms can be described as a collection of microbial cells organized within the extracellular polymer matrices, and are mostly associated with flowing systems, but can also occur as aggregates. Within the biofilm matrix, bacteria produce extracellular polymeric substances (EPS), which assist in improving the survival success of the bacteria.

The composition of EPS can be highly variable, but typically includes a variety of sugars, uronic acids, salts, DNA, and proteins. EPS are involved in many different functions in the biofilm, including increased resistance against antimicrobial agents (Dudman, 1977; Tago and Aida, 1977; Costerton *et al.*, 1981; Nguyen and Schiller, 1989; Decho, 1990). The cells within the biofilm have different degrees of resistance to biocides. This could be due to the fact that cells deep within the biofilm matrix grow at

substantially slower rates than those growing at the surface (Costerton *et al.*, 1987; Brown *et al.*, 1990; Gilbert *et al.*, 1990).

Growth of microbial cells within biofilms and subsequent EPS production depend on the availability and type of nutrients. A key factor being the availability of a suitable carbon and nitrogen source, depending on which of the available nutrients is rate limiting to the system in question. It seems possible that the lower the nutrient concentration, the greater the stimulation to produce EPS. This phenomenon was shown in the work done by Hattori and Hattori (1976) where the effect of low/diluted nutrient availability was shown, where the growth of bacteria was enhanced by their settling on a solid surface, showing a relation to the accumulation of nutrients on the surface due to adsorption to bacterial growth. This was also reported following work done on copiotrophic bacteria confronted with stressful conditions, i.e. nutrient limitation. Work done by Dawson *et al.* (1981) and Marshall (1988) showed that cells generally become more adhesive in such adverse conditions. In a study by Dawson *et al.* (1981) the mesophilic marine *Vibrio* DW1 was exposed to starvation conditions, as mentioned above. *Vibrio* DW1 responded to these conditions by increasing in cell number, decreasing in cell size and increasing adhesiveness. Marshall (1988) found that the cells become smaller and generally more adhesive when confronted with conditions of decreased nutrient availability.

As previously stated, EPS provide protection against unfavorable conditions, including biocides. Although various mechanisms of resistance have been proposed, much of the resistance among biofilm-associated cells to biocides have been attributed to a reaction-diffusion limitation of the passage of biocides across the glycocalyx (EPS) (De Beer *et al.*, 1994; Huang *et al.*, 1995). The use of biocides is often an essential procedure during water treatment to prevent the build-up of microorganisms in the form of biofilms. However, for various reasons, such as health and environmental concerns, biocides should not be used in excessive doses (Jones and Bartlett, 1985). Ideally, a water balance should be maintained throughout the purification procedure. This implies that conditions such as pH, mineral concentration, total alkalinity, calcium hardness, total

dissolved solids and temperature of the water, should be maintained at desired levels. A large variety of biocides are currently being used in water treatment, including oxidizing biocides such as chlorine, bromine and ozone. Of this group, chlorine is the most commonly used disinfecting agent. Ozone, according to Wyatt (1993) is a more powerful oxidizing agent than chlorine or bromine. The strong biocidal characteristics of ozone are due to a combination of its ability to diffuse through biological membranes and its high oxidizing potential (Hunt and Mariñas, 1997). One other advantage is that it does not produce any residual activity in water (Wyatt, 1993).

Most research on water systems has been performed on planktonic populations, even though it is the biofilm, or sessile organisms, that are often the major agents responsible for biofouling and biocorrosion. Payment *et al.* (1988) studied the incidence of bacterial indicators in two treated water filtration plants and their associated drinking water distribution systems. In the distribution systems, bacterial regrowth was observed at all sampling sites. Burke *et al.* (1984) found post-treatment increases in cell numbers of *Aeromonas* spp. in distribution systems that correlated to a decrease in free chlorine levels, indicating that exposure to sub-lethal biocidal concentrations does not prevent regrowth. It is thus necessary to study the biocidal effect of chemicals on biofilms, especially because there is a need for improved methods to control microbial growth in water used for industrial and domestic purposes. This may include the development of new, or the optimization of existing control programs. Furthermore, because the focus was traditionally on planktonic (free-floating) cells, there is a lack of methods to assess the efficiency of biocides against attached cells.

The goal of this research was to study the potential role of cells within the biofilm matrix in the survival success of the community in which they occur, specifically their contribution to the communities' ability to resist environmental changes, due to the addition of biocides. Model laboratory systems were used to evaluate the efficacy of the oxidizing biocides ozone and chlorine in the control of biofilm development by heterogeneous microbial communities. Ozone was produced by means of anodic

oxidation of water. It was anticipated that this method should enable the production of a wider range of ozone concentrations than the conventional (silent discharge) method.

The hypothesis to be tested was whether ozone, because of it's oxidizing properties, would in addition to the killing of planktonic cells, also disrupt and penetrate biofilms followed by the effective killing of biofilm-associated cells. The specific objectives included:

1). To design a model continuous flow cell system for the cultivation of microbial biofilms.

2). Show the efficacy of ozone as a biocide against planktonic and particularly biofilm populations, over different time periods, as well as the effect of different ozone concentrations.

3). Show the effect of ozone on planktonic- and biofilm populations between batch culture system with the use of a biofilm growth device.

4). Determine whole-community responses to biocide treatment, using Biolog™ and T-RFLP techniques.

CHAPTER 2. LITERATURE REVIEW.

2.1.1. Occurrence of problem microbes in water.

Microbes that are detrimental to water quality, and also cause water-borne diseases, include many bacteria, fungi, viruses and protozoa. These include, among others *Cryptosporidium oocysts*, *Giardia* cysts, *Escherichia coli, Salmonella spp.*, *Aeromonas* spp., *Pleisomonas shigelloides*, *Campylobacter spp.*, and *Legionella spp.* Illness due to water-borne bacteria most often results from ingestion of contaminated water or seafood, which allows entry of these pathogens to the gastrointestinal tract. In a summary prepared by Levy *et al.* (1998) for the Center for Disease Control and U.S. Environmental Protection Agency, the incidence of waterborne-disease outbreaks were recorded in the United States. Twenty-two outbreaks were reported in thirteen states. From these 22 outbreaks, 63.6% were caused by microbial outbreak. *Giardia lamblia* was responsible for about 9.1% of outbreaks. *Escherichia coli, Pleisomonas shigelloides* and a small round structured virus were implicated for 4.5%, each. *Giardia lamblia* and emerging protozoan pathogens such as *Isospora, Cyclospora* and *Microsporidia spp.* are, according to Curry and Smith (1998), responsible for traveler's diarrhea and water-borne and food-borne outbreaks of disease. *E.coli* 0157 was first recognized as a human pathogen in the late 1970's, and was implicated in 1982 in the large food-infection outbreak in a fast food restaurant in North America (Riley *et al.*, 1983). *E. coli* has the ability to cause a large number of diseases, ranging from mild diarrhoeal illness, to hemorrhagic colitis, hemolytic uraemic syndrome (HUS), and in some cases death. Because *Salmonella* is the main cause of food-borne and water-borne illnesses worldwide, it is important to control and prevent this organism from contaminating drinking water and fountains. *Salmonella enteritidis* is especially responsible for this contamination. Molinero *et al.* (1998) showed that *Salmonella ohio* (*S. ohio*) outbreaks were caused by the consumption of *S. ohio* contaminated drinking water. *Pleisomonas shigelloides* is an inhabitant of surface water, and is associated with warm water. Tsukamoto *et al.* (1978) showed that two epidemics of diarrhoeal diseases could possibly have been caused by *P. shigelloides.* Of the 2141 persons who stayed at the youth center of Toyono, Osaka, 978 suffered acute diarrhea in 1973.

In 1974 another outbreak occurred among employees at an electrical instrument company in Moriguchi City near the central area of the Osaka prefecture. Twenty-four out of 35 individuals participating in a sightseeing tour suffered from acute diarrhoea. In a serosurvey (test of blood serum, e.g. for the presence of a virus) conducted by Taylor *et al.* (1995) it was determined whether canoeists had a higher seroprevalence to hepatitis A, Norwalk viruses and *Schistosoma* spp. than non-canoeists in South Africa. They found no significant association between canoeing and antibody response to hepatitis A and Norwalk viruses. They did, however, find a significant association between canoeing (including contact with, and possible intake of the water) and the antibody response to *Schistosoma* spp.

A major problem experienced in South Africa is the regular outbreaks of cholera. Health officials from the World Health Organisation (WHO) first identified the problem in the Kwazulu-Natal region stemming from the townships, where access to a potable water supply of acceptable quality is almost impossible and treatment facilities inadequate. Rivers are the main source of water for cooking, cleaning and drinking, and if this supply is contaminated, the disease can rapidly spread. Cholera, an acute intestinal disease, causes the infected person to develop diarrhoea, which could lead to severe dehydration, if not treated. Fifty-eight countries have officially reported to WHO a total of 184311 cases and 2728 deaths. The overall case-fatality rate (CFR) has dropped to 1.48%, compared with 3.6% in 2000. This low CFR reflects the very low CFR of 0.22% observed in South Africa during the cholera outbreak that accounted for 58% of the world total of cases. CFR remained high in vulnerable groups in high-risk areas, and rates of up to 30% have been observed (World Health Organization, 2002). The American Red Cross reported that the key to stopping or controlling the outbreak of the disease would be the establishment of clean water sources, especially in rural areas where a communal water supply is used, preventative education and better sanitation (http:// www.redcross.org/news/in/health/010312cholera2.html).

2.1.2. Biological fouling resulting from bacterial build-up.

Biofouling can be defined as the deposition of an unwanted slimy microbial matrix on the surfaces of pipe walls, ship hulls, etc. (Flemming, 1996). Biological fouling is a problem found in water pipes and industrial cooling water systems, as well as on many submerged water systems. The deposition of the slimy matrix, or biofilms, can cause substantial problems (Epstein, 1981). Problems associated with biofouling includes costly cleaning measures, biocide consumption, shutdown and larger plants to compensate for the loss in performance. Flemming (1987) showed that biofilms could clog ion exchangers and other porous materials by increasing friction resistance. A few non-biocidal products for the control of fouling have been commercialized due to the increasing number of restrictions on the use of biocides (Flemming, 1996). Tributyltin coatings proved to be more effective over the long term than copper plating. Tributyltin coatings, however, have proved to be extremely toxic and can be accumulated in several organisms, poisoning the food chain. Tributyltin is therefore hazardous to the environment and the ecosystem (Flemming, 1996).

If a problem of microbial origin is suspected, a three-step program may be followed: identification of microbial participation, sanitation, and the prevention of further microbial growth. These steps can be carried out using oxidizing or dispersing chemicals, while brushing, flushing, or similar physical techniques are useful for the second step. To prevent further growth of microorganisms a single technique will often not be sufficient, but rather a series of events, or techniques. Another consideration is the removal of biodegradable material from the water phase if possible, as this represents potential biomass (Flemming, 1996). The existing problems with biofouling processes, i.e. the increasing costs, man-hours, and constant monitoring only reiterates the fact that improved measures against biofouling are needed. The use of a biocide that could fulfill all, or most of the requirements mentioned above, would be advantageous.

2.2.1. Biofilm growth.

Biofilms are a collection of microbial cells organized within extracellular polymer matrices, and are associated with flowing systems, but have also been found to be present in non-flowing water. Microbes generally form biofilms on any surface exposed to non-sterile water or other liquids. They generally occur at interfaces such as solid/air-interfaces, inert solid/liquid- interfaces, and solid nutrient/liquid interfaces (Gilbert and Allison, 1993). Biofilms can be found in a variety of environments and can affect many aspects of our lives. They are, for example found in lung infections, indwelling medical devices, and soft- tissue infection (Gilbert and Allison, 1993).

Biofilms have been found to protect microorganisms from excesses of oxygen concentration, redox potential and biocide treatment (Keevil *et al.*, 1993). The use of biofilms enhance the adaptation processes by permitting a high diversity of microorganisms to develop in the presence of a broad range of micro environmental conditions; promote interactions and genetic exchange between different species; favor sequential co-metabolic transformation of compounds; allow sorption of primary and secondary carbon and energy sources, sequestering them from the cells, but releasing them for subsequent degradation as solution. It has been shown that biofilms sequester toxic organic molecules, resulting in the development of concentration gradients that may allow communities to tolerate and degrade higher concentrations of toxic organic molecules than pure cultures. Bioconcentration of carbon and energy in exopolymers may also provide functional advantages at extremely low nutrient concentrations (Wolfaardt *et al.*, 1995). EPS (Fig. 2.1) may provide for controlled cross feeding between populations; and growth in biofilms also provides refuge from environmental stresses (e.g. UV, predation, pH and nutrient limitation). In addition, these communities may be stable and competent over substantial time periods providing long-term access for bacteria to zones of proliferation and survival (Bouwer and McCarty, 1982; Namkung and Rittman, 1987; Characklis and Marshall, 1990).

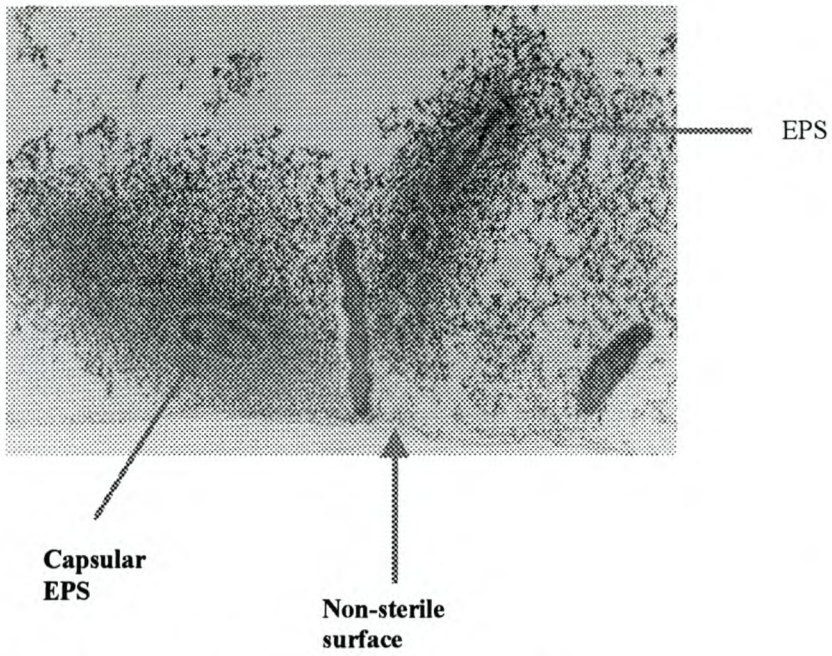


Figure. 2.1. TEM micrograph of a biofilm showing cells surrounded by EPS.

2.2.2. Stages in biofilm development.

Biofilm development is initiated when cells attach to a substratum, and under favorable conditions begin to grow, as illustrated by Dirckx (2000). This substratum can be any material in contact with a non-sterile liquid. Initially, there is reversible adsorption of microorganisms followed by the irreversible attachment. Various groups, including Meadows (1971), Lawrence *et al.* (1987), Marshall (1988) as well as Power and Marshall (1988) showed that cells attach to surfaces by a portion of the cell or flagellum during reversible attachment, after which they may either be detached, or irreversibly attach. Lawrence *et al.* (1987) explained that the reason why all cells do not become irreversibly attached could be due to the fact that the cells will firstly evaluate the potential attachment sites chemically through the use of chemoreceptors. Marshall (1988) found that the presence of bound stearic acid determined whether the motile surface-associated phase of *Psuedomonas* JD8 took place. The irreversibly attached bacteria will then grow and divide. According to a study by Lawrence *et al.* (1989)

Rhizobium spp. attach to surfaces and release daughter cells into the bulk phase. The motile daughter cells of *Rhizobium spp.* were released, reattached somewhere else on the surface and divided to form their own daughter cells. The most commonly observed colonization strategy is the formation of colonies or cell aggregates. Some surface bound cells can detach, migrate and in most cases, reattach at a suitable site. This process was termed by Lawrence and Caldwell (1987) as recolonisation. Within the biofilm matrix, bacteria produce extracellular polymeric substances. EPS production aids in the attachment of other organisms to the biofilm. According to Costerton *et al.* (1978) some bacteria form special polysaccharides in natural environments with which they adhere to surfaces. EPS provide an interface between the cell and the external environment, influencing rates of chemical exchange, availability of nutrients, and facilitating the creation of microniches (Lawrence *et al.*, 1995).

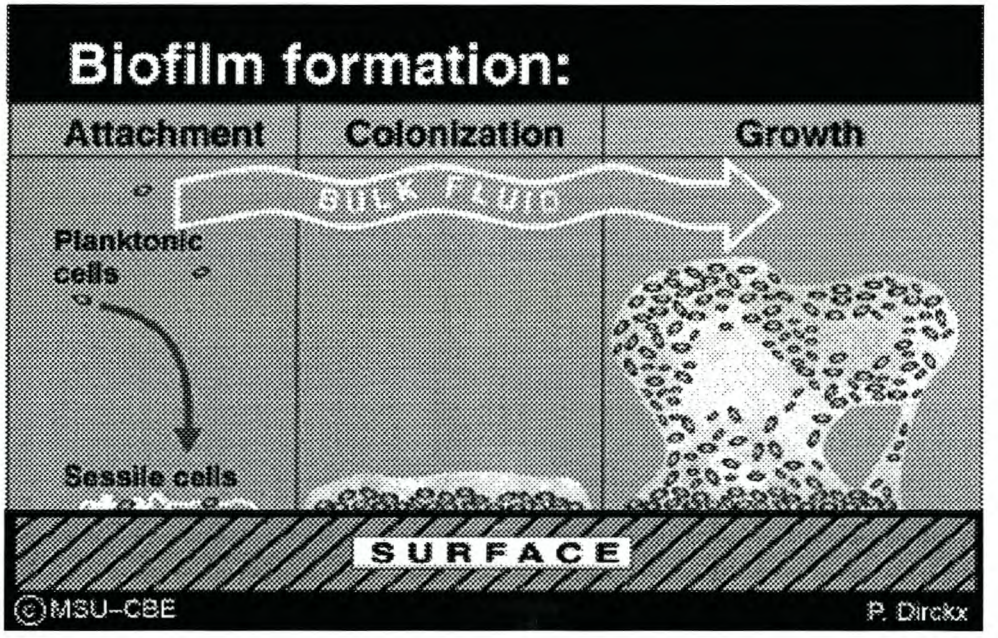


Figure 2.2. Representation of the attachment of biofilm organisms to a non-sterile surface and the subsequent growth increase to a mature biofilm. Adopted from http://www.erc.montana.edu/CBEssentials-SW/bf-basics-99/bbasics-01.htm.

2.2.3. The role of extracellular polymeric substances in survival of biofilm organisms.

EPS improves microbial competitiveness and reproductive success. These polymers play an important role in microcolony development, through the attachment of microorganisms to the surface and one another in the formation of microcolonies. It also protects bacteria within the biofilm from predation. Venosa (1975) showed that EPS act as a protective barrier against *Bdellovibrio bacteriovirus*, amoeba and bacteriophage. Studies by Giwercman *et al.* (1991) and Brown and Gilbert (1993) have shown that EPS afford resistance to biofilm microorganisms against antimicrobial agents by reducing the diffusion coefficient, the accumulation of protective agents within the EPS, or the sorption of the agent or agents to the EPS.

2.3. Devices used to evaluate biofilm communities.

In work done by Brown *et al.* (1988) to improve the understanding of the growth and development of biofilms, microorganisms were initially studied in batch culture systems. In an attempt to achieve a reproducible system that would facilitate sampling and the study of biofilm formation at different growth rates, Keevil *et al.* (1987), developed a chemostat system to model the attachment of oral bacteria, and subsequent plaque formation to acrylic surfaces immersed in steady state cultures, where the effect of biofilm growth at different time periods were evaluated. Lappin-Scott and Costerton (1989) attached a Modified Robbins Device (MRD) to a chemostat. A MRD can be used to study biofilms in areas as diverse as medical-, environmental- and industrial systems. A MRD is constructed of a long Perspex block, with a rectangular lumen containing evenly spaced sample ports with polypropylene connectors at either end for attaching the tubing from the culture vessel. The sample studs are inserted into the sample ports. MRD can be attached to the chemostat on the effluent line and the planktonic population pumped out at a flow rate controlled by the dilution rate, through the MRD and over the surface discs. These disks can then be examined microscopically or the cells can be removed from the disks and enumerated using conventional culturing techniques.

Other monitoring devices in use include the roto torque, flow cells, the Pedersen device and the constant depth film fermentor (CDFF).

2.3.1. The roto torque system.

The roto torque, developed by Kornegay and Andrews (1967), from the rotating annular reactor, consists of two cylinders constructed from Perspex, or Plexiglas with the inner cylinder rotating at a constant rate. The stator, or inner cylinder, is fitted with removable glass slides, which is used for biofilm cultivation. Rotation of the inner cylinder causes the culture medium to flow over the glass slides in a controlled, non-turbulent manner. An online torque monitor is used to monitor drag forces on the inner cylinder.

2.3.2. Constant depth film fermentor (CDFF).

A constant depth film fermentor (CDFF) is a system in which biofilm growth and film formation is controllable and reproducible, through manipulation of the nutrient- and gas supply (Wimpenny *et al.*, 1993). Wimpenny (1988) compared the CDFF with liquid gradient systems, such as the gradostat and the Herbert device, and concluded that the CDFF is a better representation of the more steady-state growth model that occurs in the natural environment. In such a system, as in nature, the culture system reaches a dynamic equilibrium, which encourages the proliferation of organisms at a relatively constant state (Wimpenny *et al.*, 1993). The film grows on the surface of a film plug located in a removable film pan. The latter rotates beneath a scraper and removes any excess film that forms (Wimpenny *et al.*, 1993).

2.3.3. Pedersen's device.

The Pedersen's device (Pedersen, 1982) consists of a closed unit through which water is diverted. Inside the unit is a coupon holder positioned in such a way to ensure even flow of the water over the coupons' surfaces. An easily removable lid allows routine sampling and replacement of the coupons.

2.3.4. Flow cells.

Flow cells (Caldwell *et al.*, 2002) are made of Perspex and contain a series of parallel channels to allow replication. A coverslip is fixed over the channels with silicone adhesive. Silicone or tygon tubing is cemented on either side of each channel to serve as influent- and effluent lines. Media is pumped to the channels through the influent tubes, along the channels of the flow cell, with the effluent, or waste pumped out through the effluent tubes, and collected in waste containers. Flow cells with various design modifications are available and compatible with different microscopy techniques. For example, Korber *et al.* (1997) incorporated microcrevices in the flow cell perpendicular to the direction of flow to evaluate the contribution of surface topography on biofilm behaviour. In the case of the study performed by Wolfaardt *et al.* (1994) the use of the flow cell was advantageous as the multi-channeled flow cell enabled simplified handling and experimental replication when biofilms grown on different surfaces were compared.

2.4. Biocide treatment.

Biocides are used in water systems to prevent the build-up of microorganisms. Different sanitizers, including quaternary ammonium compounds and oxidizing biocides are used as disinfectants. Biocides used in recreational systems, includes mainly; ozone-, chlorine- and to a much lesser extent, bromine compounds. Jones and Bartlett (1985) proved that excessive use of biocides, especially bromine, causes dermatoses in bathers, therefore bromine has not widely been used in swimming pools and is not acceptable for use in spas. Chlorine compounds include; sodium hypochlorite, dichloroisocyanurate, calcium hypochlorite and chlorine dioxide gas. Bromine compounds include elemental and bromo-chloro-dimethylhydantoin. Ozone is often used in combination with chlorine and bromine. Table 1 shows biocidal use in swimming pools and spas.

2.4.1. Treatment by chlorination.

The most commonly used disinfectant, chlorine, may be present as free available chlorine in the form of hypochlorous acid (Brungs, 1973). Hypochlorous acid dissociates into hypochlorite- and hydrogen ions, depending on pH, with hypochlorous acid being a much more effective biocide than hypochlorite ion (Seyfried and Fraser,

1980; Edlich *et al.*, 1988). Because these two ions work best at pHs between 7 and 8, the recommended pH range for swimming pools and spas, according to the Swimming Pools and Allied Trade Association (1986) and the Pool Water Treatment Advisory Group (1990), should be between 7.2-7.8. Willes *et al.* (1993) and others found that chlorine use in water systems has some adverse effects on the quality and the safety of the water. The production of trihalomethanes which were found to be carcinogenic, and other by-products cause adverse effects on the environment and human health.

Chloramines, according to Penny (1991) are formed through the combination of hypochlorous acid and urea, which is derived from sweat and urine. Chloramines are responsible for the chlorine smell. Due to thermal decomposition, more chlorine is required at higher temperatures. Although monochloramine is a weaker biocide than chlorine, the use of monochloramine instead of chlorine, will eliminate the formation of chlorinated chemicals, and therefore minimise toxicological risks. The study by Merkens (1958) concluded that free chlorine was more toxic than chloramines and residual chlorine more toxic at lower pH (6.3 vs. 7.0), because more free chlorine was present at the lower pH. In a study done by Samrakandi *et al.* (1997) the efficacies of chlorine and monochloramine (combined chlorine), against biofilms were examined. Results showed that monochloramine was more effective than chlorine, due to monochloramine not reacting with the sugars in the EPS (Jacangelo and Olivieri, 1985), while chlorine could be retained in the EPS.

Table 1. Biocides used in recreational waters adopted from T. D. Wyatt, 1993

Biocide	Swimming Pool	Spa
Chlorine compounds		
Sodium hypochlorite	+	+
Sodium dichloroisocyanurate	+	+
Calcium hypochlorite	+	+
Chlorine dioxide gas	+	-
Bromine compounds		
Elemental	+	+
Bromo-chloro-dimethylhydantoin	+	+
Ozone		
Ozone + chlorine	+	-
Ozone + bromine	-	+

2.4.2. Treatment by bromination.

Bromine dissociates into hypobromous acid and the hypobromite ion. Penny (1991) stated that due to liquid bromine's possible toxic effects to humans, it should not be used as a disinfecting agent. Bromine can be used in other applications, such as water treatment, slime control, pulp and paper refinement, etc. The biocide, Biobrom C-103 can be used for the smaller and medium sized systems, in recirculating cooling towers, as a paper slimicide and for non-marine uses in enhanced oil recovery systems (http://www.deadseabromine.com).

2.4.3. Treatment using enzymes.

In a study done by Rigaud *et al.* (1952) it was shown that enzymes may be used in association with antibiotics in the enzymatic release of microorganisms. The enzymes, in effect serve as catalysts. These catalysts hydrolyze the polysaccharides within the EPS structure, in order to 'release' bacteria from the biofilm matrix, thereby increasing the bacteria's sensitivity to the antibiotics, or disinfectants. Below are some commercially available enzymes that show promise for use to disrupt biofilms.

Lysozyme is widely prepared from egg whites. The commercial preparation of egg white is found in the form of a crystalline white powder, widely used in wine to control

lactic acid bacteria. Lysozyme is known for it's bactericidal properties against Gram-positive bacteria and its action on certain Gram-negative bacteria. Lysozyme degrades the cell wall of Gram-positive bacteria such as *Oenococcus, Pediococcus,* and *Lactobacillus*, at 250 - 300 ppm (0.94 - 1.10g/gal) (http://www.scottlab.com). Brisou *et al.* (1976) showed the destruction of *Streptococcus* cell walls isolated from patients using lysozyme.

Pullulanase is an enzyme commercially prepared from *Enterobacter aerogenes* and is used to attack certain substrates (Brisou *et al.*, 1995). Pullulanase hydrolyzes substrates such as starch and starch-related compounds in the production of corn sweeteners, baked goods, and alcoholic beverages (Messaoud *et al.*, 2002). Lipases, chitinases, and many other enzymes can be used in the destruction of cell walls, or adhesins.

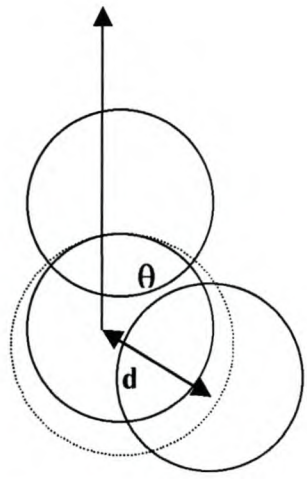
Commercially-available proteases include bromelain from pineapples, ficin from fig trees, and papain from papayas (*Carica papaya*). According to Brisou *et al.* (1995) these hydrolases act on casein and for this reason are indicated for the release of microorganisms attached to dairy products, or equipment used in the production of dairy products.

Papain has a bacteriostatic effect on *Staphylococcus aureus* by acting directly on the substrates, freeing the bacteria and thereby making them more sensitive to antibiotics (Rigaud *et al.*, 1952).

Collagenase acts on dead tissues, cellular debris, pus, and certain cellulose- or chitin-based structures in the walls of the body, thus freeing a large number of attached or buried microorganisms that are difficult to remove (Brisou, 1995).

2.4.4. Treatment by ozonation.

Ozone (O_3) is a triatomic gas comprised of three oxygen atoms (fig. 2.3). Ozone is a pale-blue gas with a peculiarly pungent smell. Ozone (O_3) is produced by the reaction of an oxygen atom (O) with molecular oxygen (O_2).



θ = 116.5°
d = 1.28 Å

Figure 2.3. Atomic model of an ozone molecule adopted from http://www.personal.psu.edu/users/d/e/der145/page1.html.

According to Null (2000), ozone is a powerful oxidant, capable of killing a wide spectrum of microorganisms including viruses (http://www.garynull.com), fungi, cysts, mildews, and spores, apart from the fact that it readily kills bacteria (http://www.personal.psu.edu/users/d/e/der145/page1.html). It is therefore widely applied in water treatment processes in waterworks (Lehtola *et al.*, 2001). Kim *et al.* (1999) showed that ozone inactivates numerous bacteria that include Gram-negative and Gram-positive, as well as vegetative cells and spores. Ozone has been widely used in medical applications. The formation of ozone is known as an endothermic reaction with large and unfavorable entropy. Commercially, most ozone is produced by the "silent electric discharge process", which involves passing air or oxygen through an intensive, high frequency alternating current (AC) (Fig. 2.4.2) electric field (Bessarabov, 1999c). The conventional corona discharge method of generating ozone has some shortcomings. These include high capital costs and the requirement of an external source of dry oxygen. It also produces a relatively low ozone concentration. Typical values of the ozone concentration are 2% wt and about 15% wt, for a corona discharge with an air- and oxygen feed, respectively. For small-scale generators, the practicalities and the economics of the operation mean that air is generally used. In this process, extensive

drying is required for a stable operation, which introduces additional costs and concerns about reliability. In the case of using the corona discharge method, with an air feed, the following reactions occur:

$$O_2 + N_2 \rightarrow 2NO$$
$$2NO + O_3 \rightarrow N_2O_5$$
$$N_2O_5 + H_2O \rightarrow 2HNO_3$$

These side reactions limit the use of air in the production of ozone for water treatment. Safety is another issue, which needs to be considered when manufacturers are working with high-voltage equipment.

Ozone can also be produced photochemically. In this case, the formation of ozone takes place when oxygen is exposed to ultraviolet (UV) light with a wavelength of 140 - 190 nm. The shortcoming of this method include low quantum yield of ozone formation from oxygen, compared with the relatively high quantum yield of phytolysis of ozone. This results in a low concentration of ozone being obtained. The typical value for UV ozone generation is 0.2% wt. Except for small-scale uses, or synergetic effects, UV photochemical ozone generation technology has not been met with a high demand.

Ozone can be generated electrochemically (fig. 2.4.1) by anodic oxidation of water:

$$9H_2O \rightarrow 6H_3O^+ + 6e^- + O_3$$

Although the use of this electrochemical reaction for generating ozone has been known for a long time, a system that would have the potential for being sufficiently attractive as the basis for a commercial generator of gaseous ozone of high concentration has not been developed (Bessarabov, 1999c).

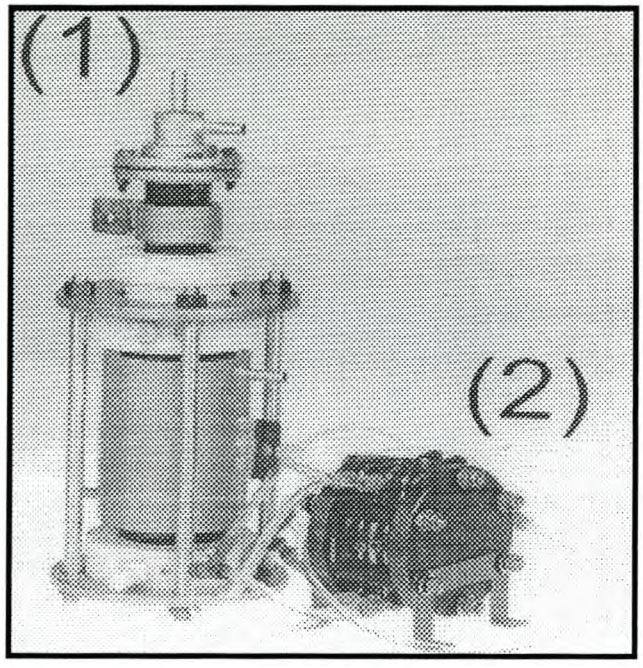
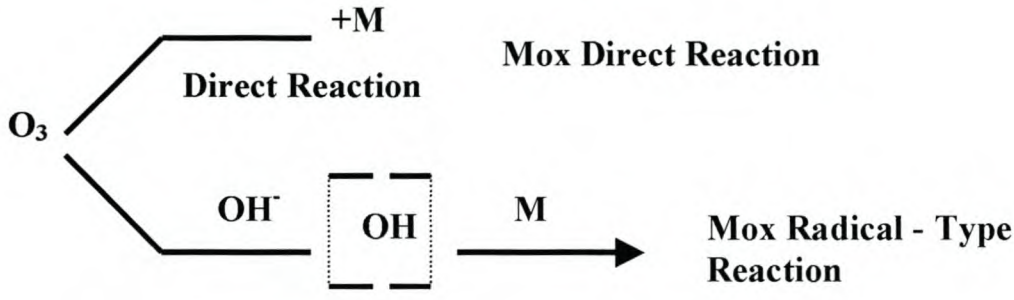


Figure 2.4. (1) High-concentration ozone (HCO) electrolytic generator for generating ozone with concentrations of about 18 - 20% wt.
(2) Low-concentration ozone (LCO) electrolytic generator for generating ozone with concentrations of about 3.5% wt.

Ozone dissociates into oxygen (O_2) and O^-. According to Hoigné and Bader (1976, 1978) ozone may in aqueous solution act on various compounds in the following two mechanisms: i) by direct reaction with molecular ozone and ii) by indirect reaction with the radical species that are formed when ozone decomposes in water. Ozonation is a commonly used technique for removing pathogenic microbes, taste and odor from water (AWWARF, 1991). Hargesheimer and Watson (1996) reported an alteration in fishy odors associated with phytoplanktonic blooms in drinking water by the combination of granular activated carbon with ozonation for removal of particulates, color, taste and odiferous compounds. Ozone, however, has a very short half-life, and should be generated on-site. Furthermore, transport of it is dangerous to humans, because liquefying ozone by compression may result in spontaneous explosions, which is why ozone should be manufactured on site (http://www.hankinozone.com/msds.html).

The activity of ozone in aqueous solution is as follows:



Ozone promotes biodegradability of dissolved organic substances. In a survey by the United States EPA it was found that ozone is capable of destroying some volatile compounds, in particular alkenes and aromatics under conditions of treatment applied to drinking water. Ozone has the quality of not only being a more powerful oxidizing agent than chlorine, but it also does not produce any residual activity in water. Due to the fact that ozone also increases biodegradability, it is more effective to disinfect water high in organic matter. This was supported by Miettinen *et al.* (1998) and Kainulainen *et al.* (1994) who stated that ozone effectively degrades natural organic matter. Ozone increases biodegradability through ring cleavage of aromatic rings, thereby giving rise to lower molecular weight carboxylic acids that are more easily biodegraded. Thus, the higher the ozone concentration utilized, the greater the degree of biodegradability.

2.4.4.1. The effect of ozone on human health.

According to Scott and Lesher (1963) as little as 0.02 to 0.04 mg/ml of ozone can be detected by man, and prolonged exposure to a concentration of 1000 mg/ml or greater, can cause death.

Atmospheric ozone produced from ultraviolet radiation combines with different nitrous oxide and sulphur products and is harmful to human health.

At concentrations as low as 15 to 82 parts per billion (ppb), ozone can cause adverse effects on humans. In Canada, it was estimated that for every 10 ppb increase in O_3 concentration, the risk of death by ozone increases by 1%, and the risk of respiratory

problems increases by 8%. People mostly at risk are those suffering from asthma and allergies. Interestingly, those who exercise outdoors during the day, especially during the summer, are also at risk due to their increased ventilation rates, and higher ozone prevalence (Kondro, 1999).

Topically, because of its haemostatic effects, ozone is used to treat burns and to stop bleeding (http://www.garynull.com). Ozone can accelerate wound healing, by activating the immune system response through the induction of enzyme production.

Ozone is a molecule that contains excesses of energy and manifests itself in having bactericidal, fungicidal and virucidal action, making ozone treatment ideal. Current ozone therapy uses a mixture of ozone and pure oxygen. Ozone research in Cuba has shown that ozone treatment is currently being deployed for wound treatments, different types of hepatitis, *Candida*, allergies and bladder infections. Other diseases or disorders treated with ozone therapy, includes herpes, arthritis, respiratory conditions, multiple sclerosis, sexually transmitted diseases and parasitic conditions (http://www.garynull.com).

Ozone is applied in the medical field in the following ways; (i) an ozone mixture is introduced into a fixed volume of the patient's blood *ex vivo*, termed as autohaemotherapy (AHT). AHT has therapeutic value in circulatory disorders, viral diseases and cancer. The therapeutic effects of ozone is that it has virucidal activity, oxygenation and increased red cell fluidity. There have been reports from AHT patients who have experienced feelings of well being lasting for a few minutes to several hours after treatment. These feelings were thought to constitute a placebo effect, a metabolic attraction, or a neuropsychiatric phenomenon; (ii) rectal insufflation is another delivery technique, where catheters are placed in the colon of the patient, and ozone gas from an ozone machine is delivered at a determined concentration flow rate. This method delivers ozone into the systemic venous system. Patients have recovered from irritable bowel syndrome, colitis and Crohn's disease almost instantly; (iii) ozone can directly be injected into veins, or muscles intravenously, but this is not recommended

(http://www.garynull.com); (iv) drinking ozonated water prepared by bubbling 1.6 to 10 mg/ml through a glass of chilled distilled water (0.56 to 4.44°C or 33 to 44°F) for 2 minutes. Ozone has been used to increase alpha interferon production, an anticancer substance.

Free radicals are molecules with unpaired electrons on their outer ring, enabling them to bind to other molecules, causing damage to cells, tissues and organs. This subsequently results in a weakening in physical vitality and damage to the neurological, cardiovascular- and immune systems. Free radicals can lead to diseases or health-related symptoms, such as aging of the skin, chronic fatigue, damage to cell membranes, cell destruction, and damage to DNA, which can cause pre-cancerous conditions. Antioxidant vitamins, such as vitamins C and –E and beta-carotene act as free radical scavengers removing them from the body after trapping them in the highly reactive singlet oxygen, thereby preventing new free radicals from being formed. Ozone can increase cellular energy by reacting with fatty acids in blood, and the cell membranes of erythrocytes. The latter reaction initiates glycolysis, increasing ATP production, which transports and releases oxygen molecules. Oxygen molecules destroy the free radicals by feeding them electrons, thereby changing their chemical structure and making them into more stable compounds. Oxygen also helps to protect cell membranes from free radical damage, preventing premature aging, cancer, heart disease, and many other degenerative conditions (http://www.garynull.com).

2.5. Evaluation of treatment programs.

In a study done by Gomella (1972) where the effectivity of ozone was compared to chlorine, a higher mortality rate of organisms were obtained when ozone was used as disinfecting agent in an environment of high organic matter content. Ozone showed a stronger and more rapid antimicrobial action against spores, faecal and pathogenic microorganisms as well as viruses. Also against the poliovirus, ozone was superior to chlorine according to Scarpino *et al.* (1972). According to Wei *et al.* (1985) and Page *et al.* (1967) it was found that chlorination of water, and food- or contact surfaces, may lead to the formation of toxic or carcinogenic chlorinated organic compounds. It is necessary to reduce chlorine concentration due to the presence of carcinogenic

trihalomethane compounds (THM's) that are formed by the reaction of free chlorine (HOCL, OCl^-) with soluble organic compounds.

Restaino *et al.* (1995) used a recirculating concurrent reactor to perform a study on the efficacy of ozonated water against various food-related microorganisms, while Kim (1998) used a batch type reaction system. In the studies it was found that death rates among Gram-negative bacteria was not significantly different after exposure at 1 to 1.5 ppm of ozone. Furthermore, the study of Restaino *et al.* (1995) found similar results with other Gram-negative bacteria. The Gram-positive bacteria, *Staphylococcus aureus* and *Enterococcus faecalis* in the Restaino study were less sensitive than *L. monocytogenes*, whereas in the batch system of Kim (1998) *Leuconostoc mesenteroides* were more sensitive than *L. monocytogenes.*

Chen *et al.* (1993) compared the effectivity of chlorine disinfection to monochloramine disinfection. They found that monochloramine disinfection was more effective in the destruction of *Pseudomonas aeruginosa* biofilm cells than free chlorine. They also demonstrated that the media used determined the rate of EPS production and the subsequent increase in resistance of cells within the biofilm to biocides. Samrakandi *et al.* (1997) showed that when *E. coli* grown on lactose, and mucoid *Pseudomonas aeruginosa* medium-grown (PAM), *P. aeruginosa* biofilms were exposed to chlorine and monochloramine disinfection, respectively, monochloramine proved to be more successful than chlorine. This could be due to the fact that according to Jacangelo and Olivieri (1985) chloramine does not react with sugars and their penetration would therefore not be restricted by the glycocalyx (De Beer *et al.*, 1994; Huang *et al.*, 1995). Biofilms expressing high levels of EPS may decrease chlorine activity, by the retention of the antimicrobial agent within the extracellular polymeric substances of the biofilm. The study by Xhu *et al.* (1996) showed attenuation of chlorine by alginate beads and subsequent decrease in biocidal activity, provided support for this contention.

Ewell (1938) stated that depending on cleanliness, a minimum continuous concentration of 0.6 to 1.5 ppm ozone was necessary to prevent mould growth on eggs kept at 0.6°C

and 90% humidity, whereas 2.5 to 3.0 ppm ozone was required to control moulds on beef that was stored under similar conditions.

Farooq and Akhlaque (1983) showed that ozone also inactivated yeasts. Yeasts appeared more sensitive than moulds to ozonation. Using a recirculating concurrent reactor, Restaino *et al.* (1995) showed that more than 4.5 log of *C.albicans* and *Zygosaccharomyces bailli* populations were killed instantaneously in ozonated water, whereas less than 1 log of *Aspergillus niger* spores were killed after 5 min. exposure.

Broadwater *et al.* (1973) reported an inactivation of *Bacillus cereus* and *Bacillus megaterium* spores, but no effect has been shown against spores of other *Bacillus* and *Clostridium spp.* A subsequent study by Foegeding (1985) evaluated the effect of ozone against *Bacillus cereus* T, *B. stearothermophilus*, *C. perfringens* and *C. botulinum* spore populations. Ozone proved to have rapid and effective sporicidal activity. It only required 1.5 mg ozone/l to inactivate 90 to 99% of *B. cereus* T spores. *B. stearothermophilus* exhibited greater resistance, where 2.2 mg ozone/l inactivated about 60% of spore populations. *B. cereus* spore populations with a removed spore coat protein were rapidly inactivated (99.995%) by 0.2 mg ozone/l, indicating the spore coat is a primary protective barrier against ozone.

Woerner *et al.* (1970) found that 5 - 10 mg/l gaseous ozone was adequate to eliminate bacteria, such as *Salmonella spp.* after a contact time of 7 min. and anthrax spores after 30 min. Kim *et al.* (1999) concluded that ozone treatment could be used to disinfect lightly polluted dairy effluent for re-use. Ito and Seeger (1980) found that post-process spoilage of canned food decreased by using ozonated water for cooling cans.

2.6. Community structure analysis.

Microbial community composition is not well described by classical microbiological techniques because cultures are often viable, but not culturable. Existing tools for these analyses have proven to be both time-consuming and laborious. In order to eliminate time as a factor in the isolation and culturing of organisms various molecular techniques

may be employed to assess microbial diversity, eliminating the need for isolation (Ritchie *et al.*, 2000). For this purpose, techniques such as polymerase chain reaction - restriction fragment length polymorphisms (PCR-RFLP), denaturing gradient gel electrophoresis (DGGE), Biolog™ substrate utilization profile analysis, fatty acid methyl esterase (FAME) analysis, and phospholipid fatty acid analysis, as well as terminal-restriction fragment length polymorphisms (T-RFLP) have been employed.

2.6.1. Biolog™ whole community analysis.

Because cells express a large number of phenotypes, an overall phenotypic analysis is needed in order to find traits common to groups of organisms, such as species (Bochner, 1989).

The Biolog™ carbon utilization technique was developed by Biolog Inc. The technique was based on the formulations by den Dooren de Jong (1926) and that of Bochner and Savageau (1977). This method shows great promise in speeding, standardising and simplifying the process of metabolic testing. The method enables the user to simply and efficiently characterize and identify microbial isolates, by examining their carbon source utilization profiles. In work done by Bochner and Savageau (1977) it was shown that tetrazolium dyes could be used as colorimetric indicators when carbon substrates are reduced by microbial metabolism. In the test, miniaturized substrate utilization test plates, i.e. micro-titre plates were used to identify, or at least classify bacterial isolates, as in the work done by Fulthorpe *et al.* (1993) and also that of Frederickson *et al.* (1991). An important function of Biolog™ technology is that it is universal and adaptable. When a substrate is oxidized, it results in the formation of NADH, which donates electrons to the electron transport chain. Biolog™ technology can therefore function independently of the electron transport chain and the anaerobic bacteria's electron transport chain mechanism can be diverted to reduce tetrazolium. Tetrazolium will be converted to a colored formazan if it taps the electrons resulting from the formation of NADH, the electron donor (Bochner, 1989). There are various types of Biolog™ microplates available, which includes, Biolog MT plates, Biolog GN/GP plates, primarily used for identification. The Biolog MT plates do not contain any carbon substrates, so that the substrates can be added later, as used by Fulthorpe and

Allen (1994) in their evaluation of Biolog MT plates for aromatic and chloroaromatic substrate utilization tests. Commercially manufactured Biolog^{TM} ecoplates contains 3 replicates of 31 different carbon sources, making up the 96 well plate. Carbon utilization in each well is monitored by a resulting purple color change (Di Meo et al., 2000).

2.6.2. Phospholipid fatty acid analysis.

Phospholipid fatty acid analysis enables the user to distinguish shifts in the phospholipid fatty acid "fingerprint or profile" of the microbial community, according to White and Findlay (1988). These workers argued that whereas DNA extraction, screening and sequencing are labor intensive and time consuming, fatty acid analysis allows one to quantify community structure without relying upon these time consuming steps or cultivation of microorganisms. This technique does not allow for identification of organisms at species and strain level, but it does provide descriptions of microbial communities based on the functional groupings of fatty acid profiles (Ibekwe and Kennedy, 1998).

2.6.3. Polymerase chain reaction - restriction fragment length polymorphisms (PCR-RFLP).

PCR-RFLP is a rapid, PCR-based genotypic method for typing microorganisms (Swaminathan and Matar, 1993; Arbeit, 1995). In this method, an initial PCR is performed where known primers to a particular region is attached. A DNA fingerprint results after running the digested amplicon onto an agarose gel followed by staining with ethidium bromide. The main advantage of this technique lies in its speed, reproducibility and in it's simplicity. Some of the disadvantages include; a requirement of prior knowledge of the DNA in the region of interest, the discriminatory power of the technique tend to differ greatly depending on the different species, loci, and restriction enzymes, and the method is generally thought of as being only moderately discriminatory. Investigators have shown that this method can be utilized not only for typing purposes, but for species identification as well (Niesters *et al.*, 1993). To distinguish between differences in the community structure of a particular microbial

community, an analysis of their genetic structure needs to be performed. For this means, use could be made of techniques such as denaturing gradient gel electrophoresis (DGGE) and terminal-restriction fragment length polymorphisms (T-RFLP).

2.6.4. Denaturing gradient gel electrophoresis (DGGE).

This technique enables the detection of differences in the melting behaviour of small DNA fragments of the sizes between 200-700 bps. DGGE also enables analysis of many fragments simultaneously on a single denaturing gel in which the direction of electrophoresis is perpendicular to that of the denaturing gradient (Gray, 1987).

2.6.5. Terminal restriction fragment length polymorphisms (T-RFLP).

T-RFLP analysis is a culture-independent molecular fingerprinting technique based on small-subunit (SSU) rRNA genes (rDNA). The technique is used to address questions about structural composition, diversity and dynamics of microbial communities (Engelen *et al.*, 1998). The richness and evenness of a community are qualitatively estimated based on the number of unique clones and the relative frequencies of the variations in the T-RFLP patterns obtained (Liu *et al.*, 1997). The main procedure for the T-RFLP technique involves the amplification of the targeted gene using standard PCR techniques. The PCR involves the adherence of 5' and 3' fluorescently tagged primers. The resulting products are then digested with restriction endonucleases and run on an automated sequencer. The restriction endonuclease should be chosen such that following digestion each labeled fragment corresponds to a different sequence variant (Bruce and Hughes, 2000). The fragment proximal to the restriction enzyme carries the fluor and is the only fragment that will be detected by the sequencer. For the study conducted by Lukow *et al.* (2000) the use of the T-RFLP analysis resulted in very complex but highly reproducible community fingerprint patterns. Also shown by Osborn *et al.* (2000), was that TRFLP analysis is a highly reproducible and robust technique that yields high-quality fingerprints consisting of fragments of precise sizes, which in principle could be phylogenetically assigned.

CHAPTER 3. METHODOLOGY.

3.1. Strain selection and growth conditions for planktonic evaluations.

An unidentified, Gram-positive, exopolysaccharide-producing, coccoid bacterial strain (AB9) was selected for all pure culture experiments. Ten percent (w/v) Tryptic Soy Broth (TSB) obtained from Biolab Diagnostic, Merck, was prepared for cultivation purposes.

3.2. Growth medium.

Unless stated otherwise, all biocide evaluations were performed using an overnight grown culture of strain AB9. For cultivation, 10% Tryptic Soy Agar (TSA), prepared from TSB and bacteriological agar, was prepared using conventional methods and placed on a shaking incubator at 30°C.

3.3. Ozone generation.

Ozone generators developed by DINAX Technologies, CC (Matieland, Stellenbosch) were used in the study (Bessarabov, 2000).

3.3.1. High concentration (18 – 20% wt) ozone preparation.

High concentration ozone (HCO) was generated electrochemically (Fig. 3.1) by the anodic oxidation of water containing a water-soluble inorganic proprietary electrolyte (Bessarabov, 2000). At the anode a mixture of oxygen and ozone is produced (reactions i and ii), while at the cathode hydrogen is produced (reaction iii). Ozone formation by anodic oxidation of water is a complex process involving the formation of various short-living intermediates (Bessarabov, 2000).

ELECTROLYTIC OZONE GENERATION REACTIONS:

Anode

$$6H_2O \rightarrow 4H_3O^+ + 4e^- + O_2 \quad E^0 = 1.23V \qquad \text{(i)}$$

$$9H_2O \rightarrow 6H_3O^+ + 6e^- + O_3 \quad E^0 = 1.51V \qquad \text{(ii)}$$

Cathode

$$4H_3O^+ + 4e^- \rightarrow 4H_2O + 2H_2 \qquad \text{(iii)}$$

3.3.2. Low concentration (0.3 -1% wt) ozone preparation using the DINAX generator.

Low concentration (0.3 – 1% wt) ozone was generated electrochemically by the anodic oxidation of water (Fig. 3.1). Low concentration ozone (LCO) was also generated electrochemically by anodic oxidation of water by the use of a perfluorinated ion-exchange membrane and a lead dioxide anodic catalyst (Bessarabov, 1999a, b, c and Bessarabov, 2000). The ozone generator used in this study was also developed by DINAX Technologies, CC. Ozone concentrations ranged between 0.3 and 1% wt.

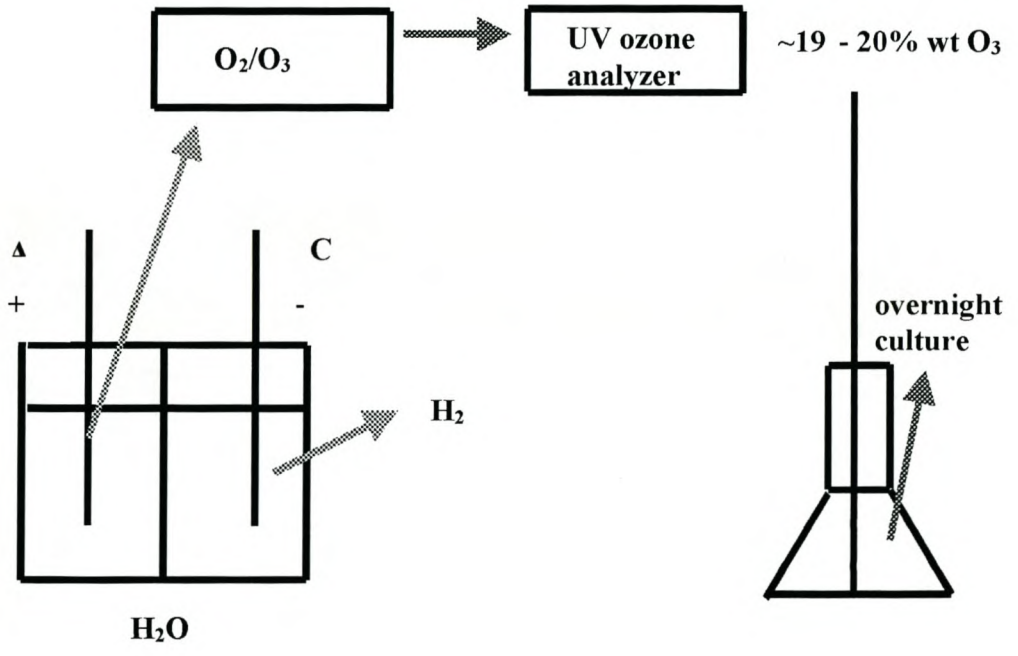


Figure 3.1. Schematic representation of ozone generator and treatment of planktonic populations.

3.3.3. Low concentration (3.5% wt) ozone preparation using a Fischer generator.

Low concentration ozone (LCO) was generated by single electric discharge using the corona discharge method. A commercially available oxygen-fed Fischer ozone generator was used to yield a low ozone concentration. The concentration of ozone yielded was in the order of 3.5% wt O_3 (Bessarabov, 1999c).

3.4. Effect of different ozone concentrations and exposure times on planktonic populations.

3.4.1. High concentration ozone (18-20% wt).

O_3 generated as described in 3.3 was bubbled directly into 300 ml of an overnight culture of strain AB9. The exposure times were 5-, 7-, 10-, and 15 min. of direct bubbling into 300 ml aliquots of the overnight culture (Fig. 3.1). Each of these flasks was incubated and samples collected after 3-, 5-, and 24 hours, respectively, and surviving cell numbers determined using the plate count technique, on 10% TSA plates.

3.4.2. Low concentration ozone (3.5% wt and 0.3-1% wt ozone).

O_3 generated as described in 3.3 was bubbled directly into 300 ml of an overnight culture of strain AB9. The exposure times were 5-, 7-, 10-, and 15 minutes of direct bubbling into 300 ml aliquots of the overnight culture (Fig. 3.1). Each of these separate flasks was incubated and samples collected after 3-, 5-, and 24 hours, respectively, and surviving cell numbers determined using the plate count technique.

3.4.3. The effect of an increased exposure time to lower (3.5% wt) ozone concentrations.

In order to evaluate the effect of exposure to an extended bubbling time period, 300 ml of the overnight culture was exposed to direct ozone bubbling for periods of 30 or 60 minutes. The exposed culture was then incubated for periods ranging from 1-, 3-, 5-, and 24 hours, respectively, and surviving cell numbers determined as described previously.

3.5. Biofilm analysis in continuous flow systems.

3.5.1. Strain selection for biofilm cultivation.

Six bacterial isolates obtained from the natural environment, were selected for their exopolysaccharide-producing properties. These strains were also selected because of their presumed survival abilities against exposure to lower concentrations of ozone. These experiments were carried out by the addition of O_3 bubbled in water to different volumes of overnight culture, e.g. to 20 ml of overnight culture, 80 ml of ozonated water was added. The same was done for 50- and 80 ml of overnight cultures, to make up a final volume of 100 ml. After different time intervals (1-, 3-, 5-, 24 hours) dilution series were prepared followed by viable counting on 10% TSA plates, where stains were selected as mentioned above due to their survival capabilities.

3.5.2. Growth medium.

Standard microbiological techniques were applied to prepare a 1% (w/v) TSB solution. This sterile medium was inoculated with the six strains mentioned above and incubated in a shaker at 30°C for 24 hours, at a speed of 100 rpm, in order to simulate conditions in the natural environment.

3.5.3. Model continuous flow system for biofilm cultivation.

A Perspex flow cell was set up (Fig 3.2). The flow cell contained a series of parallel channels to allow replication. Observations included focusing on the inside surface of the coverslip. The coverslip was fixed to the Perspex plate, using Bostik® Marine Clear Silicone Sealant. The flow cells were surface-sterilized with a 3.5% m/v sodium hypochlorite solution, after which they were rinsed with sterile distilled water. A Watson Marlow 205S peristaltic pump (Falmouth, Cornwall TR11 4RU, England) was used to continuously deliver the growth medium at a speed of 0.0024 ml/min. The same procedures were followed using the high and low ozone (O_3) concentrations, generated as mentioned above in 3.3.

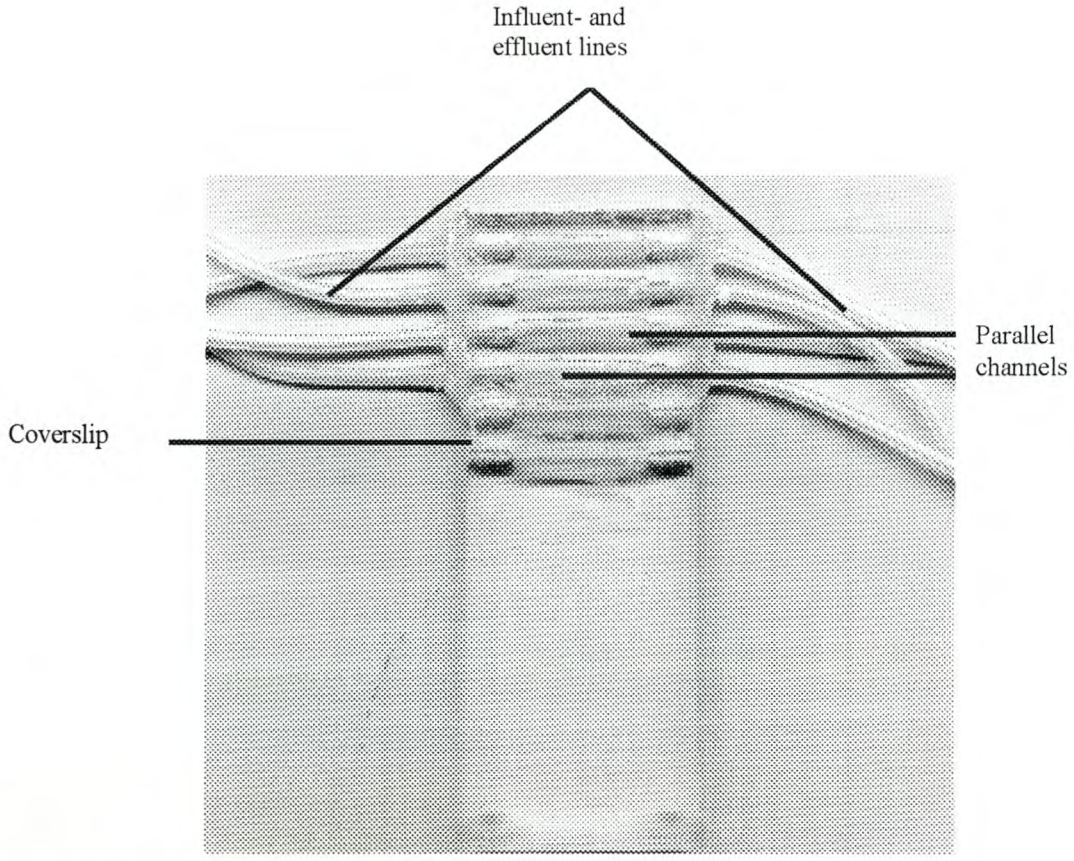


Figure 3.2. Flow cell apparatus used for the cultivation of microbial biofilms.

3.5.4. Biofilm cultivation.

Biofilms were allowed to grow over a period of weeks in the flow cells. After cultivation, the flow cells were exposed to O_3 dissolved in water, at concentrations of 3.5% -, and 18% wt O_3. The first untreated channel was designated as the control. The subsequent 3 treated channels were used as the test chambers.

3.5.5. Effect of different concentrations and exposure times on biofilm integrity.

After O_3 (3.5% -, and 18% wt O_3) exposure times of 0-, 1-, 1.5-, 2.5 and 3 hours, per respective channels, the Baclight viability probe (Molecular Probes, Eugene, Oregon, USA) was added to the channels of the flow cell, and the procedure was followed as described in 3.5.6. Evaluation was by means of epifluorescence microscopy. These

experiments were performed thrice and the results obtained were the averages of these replicate experiments. The same procedures were followed using 3.5% -, 18% wt O_3, generated as mentioned in 3.3.

3.5.6. Image analysis.

The Baclight™ viability assay is based on fluorescence. It is a single-step alternative to the conventional bacterial viability tests. Each kit contains a mixture of nucleic acid stains that rapidly distinguish between live and dead cells. A probe was inoculated into the flow cell chambers after treatment with the biocide. The multiple-fluor probe, Baclight™ contains a green fluorescing stain which penetrates both the membrane and the cytoplasm, and a red fluorescing stain, which does not penetrate the cell unless there is a loss of membrane integrity. Thus, living cells appear green, and dead cells red.

The probe was prepared by mixing 4µl of component A (Nucleic acid stain) and 4µl of component B (Propidium iodide solution), to which 1 ml of distilled water was added. The Watson Marlow 205S pump was switched off and 200µl of the Baclight™ probe was added to each of the chambers. The probe was left in the chambers for a period of 15 minutes, in order to allow the probe to react to the cells in the biofilm. After the staining period, the excess probe was washed out from the chambers, by switching on the pump once again for 5 minutes. After this period, the flow cell was viewed under epifluorescence microscopy, at an enlargement of ×60, using oil immersion.

3.5.7. Epifluorescence microscopy.

In each channel, 10 images were randomly captured along the surface of the coverslip and the percentage area covered by living and non-living biomass determined using Scion Image (http://www.Scioncorp.com). These experiments were performed thrice and the results obtained were the averages of these replicate experiments. The same procedures were followed using both the high and low ozone concentrations, generated as previously stated. The experimental procedure is summarized in Fig 3.3.

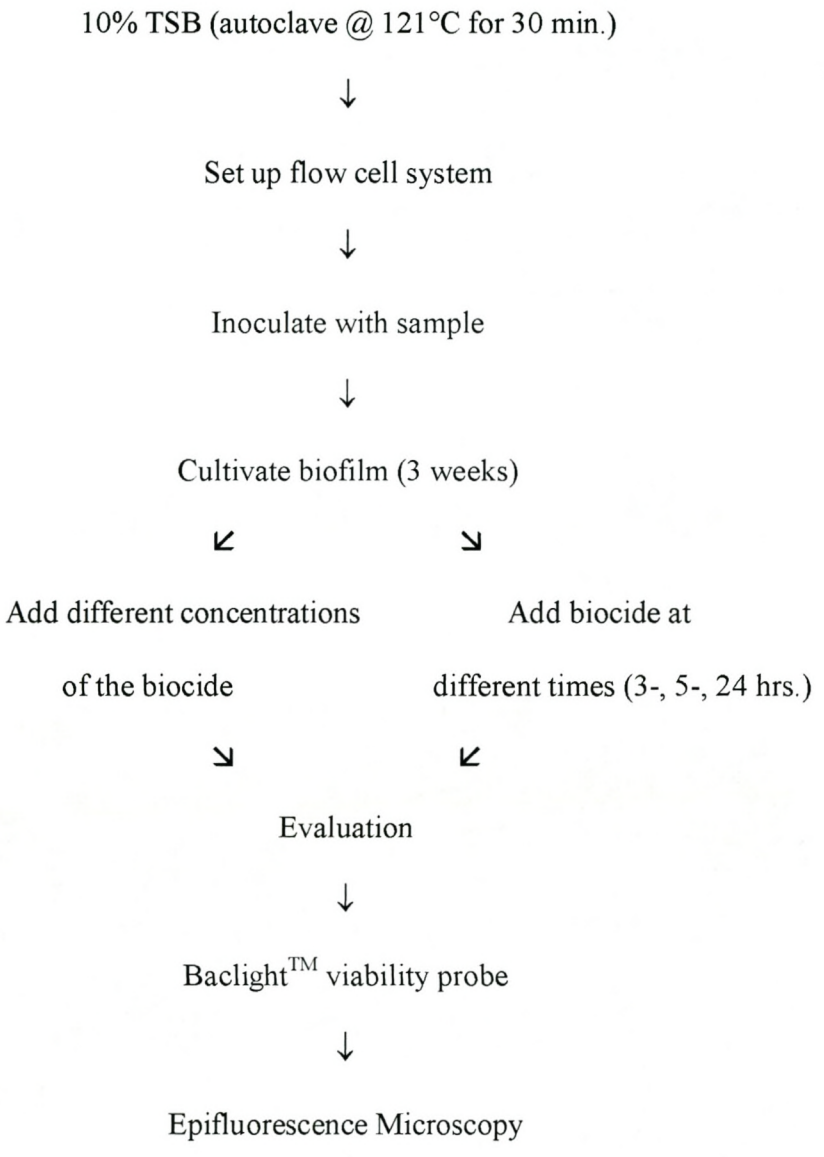


Figure 3.3. Schematic representation of method set-up for biofilm population evaluation, as explained in 3.5.

3.6. Batch culture analysis.

3.6.1. Effect of high concentration of ozone on the integrity of biofilms cultivated in a batch culture system.

The biofilms were cultivated over a period of 3 weeks, this time using a batch system. This batch culture system was set up by preparing 0.1% (w/v) TSB in a 2L container, following conventional methods. The growth medium was inoculated with a mixture of the same six organisms (table 4.1) used for the continuous culture experiments, and placed in a shaking incubator, for a period of 24 hours. The batch culture set up included glass microscope slides (76 x 26 mm, 1.0/1.2 mm thick) for the cultivation of biofilm. The microscope slides were placed in a Perspex slide holder, which is in essence an open-ended box that allowed free flow of water over the attachment surfaces. This open-ended box was fitted with 12 corresponding grooves in the top lid and bottom portions into which the glass slides were slotted. The outside dimensions of the holder were 82 x 82 x 40 mm. An elastic band was used to keep the top cover on and the glass slides in place. The batch system was placed on a magnetic stirrer, in order to ensure that the growth medium was kept uniformly distributed through the system, and the slide holder suspended in the medium (Fig. 3.4). The medium was replaced periodically (48 h interval) to sustain the biofilms on the attachment surfaces. After allowing biofilm formation on the glass slides for a period of 3 weeks, the biofilm growth generator was exposed to ozone by purging the ozone into the medium for different time periods. Samples were obtained at different time intervals, as explained in 3.6.2.

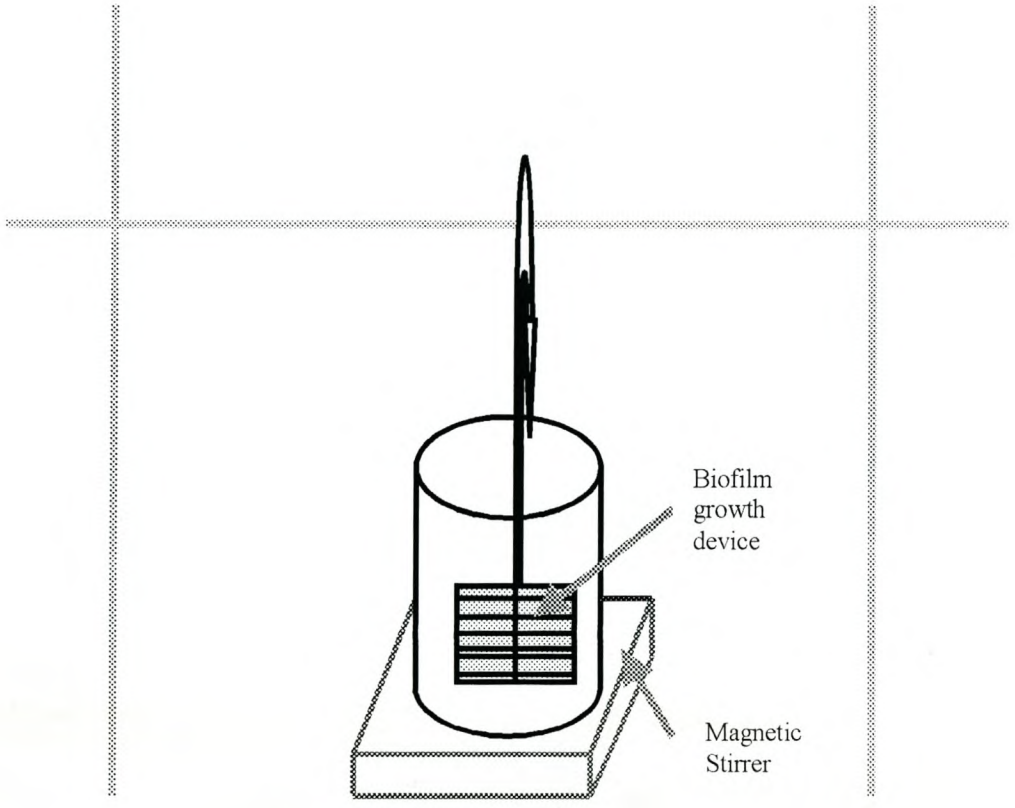


Figure 3.4. Biofilm growth generator submerged in media inoculated with six different organisms.

3.6.2. Epifluorescence microscopy and image analysis.

The Baclight™ viability probe was prepared as described before in 3.3.2.4. Glass slides were removed from the biofilm growth device at different time periods of 0 (untreated)-, 5-, 7-, 10-, 15-, 25-, 60-, 180-, and 1440 minutes (all treated with the biocide). The glass slides were cleaned on the one side to allow easier evaluation, and the other side was stained with the probe for 10 minutes, after which the probe was gently washed off with sterile distilled water. Image analysis was performed as described in 3.5.6.

3.6.3. Carbon utilization patterns of the batch populations.

After a 25 min. exposure time, samples were collected from the reservoir to determine whether the addition of the biocide influenced the population composition of the cell suspension. For this purpose, Biolog™ ecoplates were used to obtain whole-community metabolic profiles. Samples were removed at time 0h (untreated control), 25 mins, 3h, 24h, 48h, 96h and 168h. The 96-well ecoplates consist of 3 replicates, each of which contains 32 wells. Fourteen ml of the removed sample was aliquoted into a 15 ml polypropionate centrifuge tube and centrifuged at 12 000 rpm for 10 minutes, to remove any excess media before inoculation of the plates. The supernatant was removed and the pellet resuspended in 14 ml sterile Ringer's solution, of which 150µl was aliquoted into each well. The plates were incubated at 25°C for a time period of up to three 3 days before wells with positive reactions (substrate utilization) were counted. Statistica™ was used to construct dendograms to reveal changes in community structure.

3.7. Effect of chlorine on carbon utilization patterns by biofilm communities.

Experiments were also conducted to evaluate the effect of chlorine (another widely used oxidizing biocide) on biofilms. Similar to the ozone-treated biofilms, biofilms were cultivated in flow cells, but were subsequently exposed to a chlorine concentration of 4 mg/ml chlorine (total chlorine) for 30 minutes. After exposure, effluent was collected from the flow cell channels. It was assumed that after exposure and removal of the chlorine, by the flushing of the flow cell, and the associated suspended cells, the cells subsequently present in the suspended phase represented those from the biofilm phase, and therefore the biofilm phenotype. The effluent from the 4 different flow cell channels were collected and dispensed into separate 15 ml polypropionate centrifuge tubes and centrifuged at 12 000 rpm for 10 minutes. The Biolog™ technique was subsequently performed as described in 3.6.3.

3.8. Microbial genetic diversity following biocide treatment.

Analysis of microbial diversity includes DNA extraction, Polymerase chain reaction amplification and terminal restriction fragment length polymorphisms (Fig. 3.5).

3.8.1. DNA extraction.

DNA extraction was performed using the method developed by Zhou *et al.* (1996). There were a few adaptations of the method, which included:

Biofilms cultivated in flow cells were exposed to a chlorine concentration of 4mg/ml chlorine, for 30 minutes. After exposure, effluent was collected from the flow cell channels and prepared the same way as discussed in section 3.7. One ml of the resuspended pellet was aliquoted into 1.5 ml Eppendorf tubes. The Eppendorf tubes were centrifuged once again at 3000g for 3 minutes. The supernatant was removed and the pellet resuspended in 1 ml DNA extraction buffer. The Eppendorf tubes containing the resuspended solutions were then exposed to 3 freeze-thaw cycles (-70/+65°C) to break open gram-positive cells. Each freeze-thaw cycle involved keeping the cells for 15 minutes in the –70°C freezer, immediately followed by shocking these cells for 2 minutes at 65°C in a water bath. To the freeze-thawed samples, 5 µl of Proteinase K (20 mg/ml) was added, mixed well, and incubated at 37°C for 30 minutes in a shaking incubator. After incubation, 150 µl of 20% SDS was added, incubated at 65°C for 2 hours, and gently mixed every 20 minutes. Upon completion of the incubation period, the samples were centrifuged at 6000g for 10 minutes. The subsequent supernatant was then dispensed into a 10 – 15 ml polypropylene clean tube (chloroform resistant). To these supernatants, an equal volume of chloroform/isoamyl alcohol (24:1) was added and mixed well in a fume cabinet. Centrifugation proceeded at 3000g for 3 minutes, after which the aqueous layer (top) was transferred to clean Eppendorf tubes. A 0.6 volume of cold isopropanol was added to the tubes, mixed well and left overnight in the –20°C freezer. The tubes were then centrifuged at 16000g for 20 minutes, and the isopropanol decanted. The pellets were washed with 1 ml ice cold 70% ethanol, centrifuged at 3000g for 2 minutes, the ethanol decanted, and the tubes allowed to dry completely. The completely dried pellet was then dissolved in 150µl of TE (Tris 10 mM, EDTA 0.1 mM). Six µl of the DNA sample was then run on a 0.8% molecular

grade agarose # D1 -LE gel obtained from Whitehead Scientific (Brackenfell, South Africa) to ascertain whether DNA was present.

3.8.2. DNA purification.

Purification involved the use of a Microspin™ S-300 HR column DNA purification kit, obtained from Amersham Pharmacia Biotech Inc. (New Jersey, United States of America). Six µl of the subsequent purified DNA was run on a 0.8% molecular grade agarose # D1 -LE gel, before proceeding with the Polymerase chain reaction (PCR).

3.8.3. Polymerase chain reaction.

For PCR, 25 µl of a PCR mix, containing 4 dNTP's (dATP, dGTP, dCTP, dTTP) of 400 µM each, 50 units/ml Taq polymerase, 3 mM $MgCl_2$, 10X DNA buffer (pH 8.5), obtained from the Promega Corporation (Wisconsin, United States of America); 2 µl of purified DNA; as well as 2.5 µl each of forward and reverse universal bacterial primers. The forward primer (fDD2 5'CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG3' 34-mer) and reverse primer (rPP2 5'CCAAGCTTCTAGACGGITACCTTGTTACGAC TT3' 33-mer), as described by Rawlings (1995) were added to the PCR-DNA mix and nuclease-free water added to a final volume of 50 µl. The amplification process was 4 hours long, using a Perkin-Elmer, Gene Amp 2400 PCR system. The PCR reaction had an initial denaturation step at 94°C for 2 minutes, followed by 30 cycles of amplification (1 minute at 94°C, 1 minute at 57°C and 2 minutes at 72°C). This was followed by a final extension step of 72°C for 10 minutes. The subsequent amplicons were then run on a 0.8% molecular grade agarose # D1 -LE gel, to ascertain whether amplification was successful.

3.8.4. Polymerase chain reaction using labeled primers.

The purified DNA was amplified with oligonucleotide primers, fluorescently labeled at the 5'- and 3' ends with phosphoramidite dyes 6-FAM and HEX. The primers were FAM63F (3'-CAGGCCTAACACATGCAAGTC-5') and HEX1389R (5'-ACGGGCGGTGTGTACAAG-3') according to Osborn *et al.* (2000). The PCR cocktail was prepared as described in 3.8.3 the only difference was the use of fluorescent

primers. Amplification was performed as before. The amplicons were run on a 0.8% molecular grade agarose # D1 -LE gel, and the PCR products subsequently purified using Nucleospin® Extract columns obtained from Macherey-Nagel (Düren, Germany).

3.8.5. Terminal-restriction fragment length polymorphisms (T-RFLP).

Each of the PCR products was digested with the restriction enzyme, *HhaI*. Each digestion mixture consisted of 20 µl of PCR products, 1 µl of *HhaI*, 3 µl restriction digest buffer and 6 µl sterile distilled water. The digestion mixtures were incubated at 37°C for 10 hours and placed at 70°C for 15 minutes to terminate the digestion reaction. The digested DNA was mixed with a labelled DNA size standard (Rox 500) and the fragments analysed on an ABI 3100 genetic analyser (Fig. 3.5).

3.8.5.1. Whole-community profile testing.

T-RFLP analysis was utilized to compare possible shifts in community composition between the untreated controls and the biofilm community treated with chlorine – see sections 3.5 and 3.6).

3.8.5.2. T-RFLP on individual community members.

T-RFLP analysis was used to compare the dominant bacterial species pre and post treatment. A sampling procedure was used that ensured uniformity in the selection of colonies (Fig. 3.6). After the samples were collected from the effluent containers, serial dilutions were prepared, and plated onto 10% (w/v) TSA plates. The 10^{-7} plates were used for selection of suitable colonies. A petri dish sized paper disc, sectioned into four parts, each part divided into four subsequent sections were used as a template for random selection. Colonies within a selected section, were isolated (for instance only the colonies in section 3 of the different quadrants), and streaked onto 10% (w/v) TSA plates.

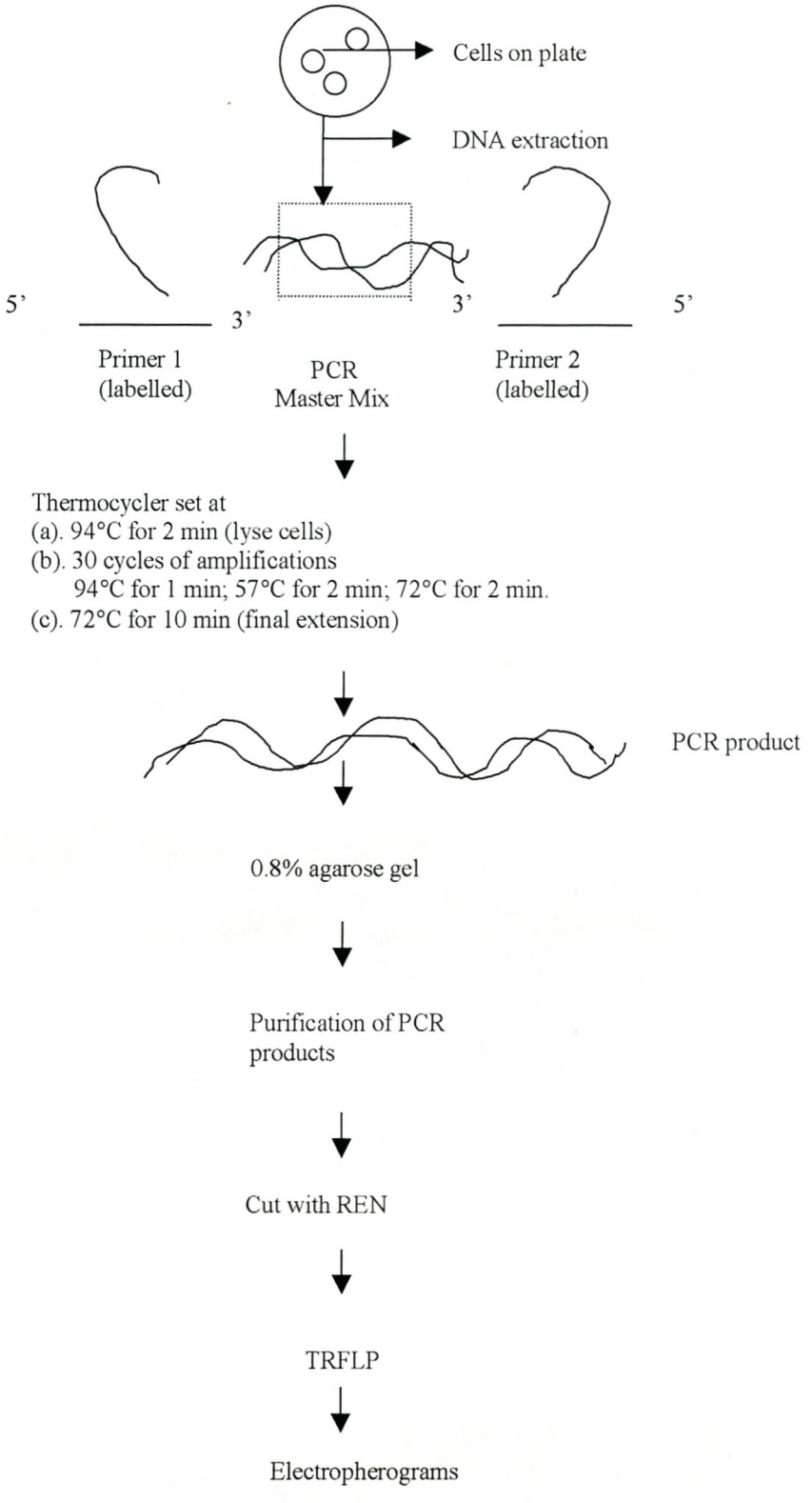


Figure 3.5. Schematic representation of the procedure followed for TRFLP analysis.

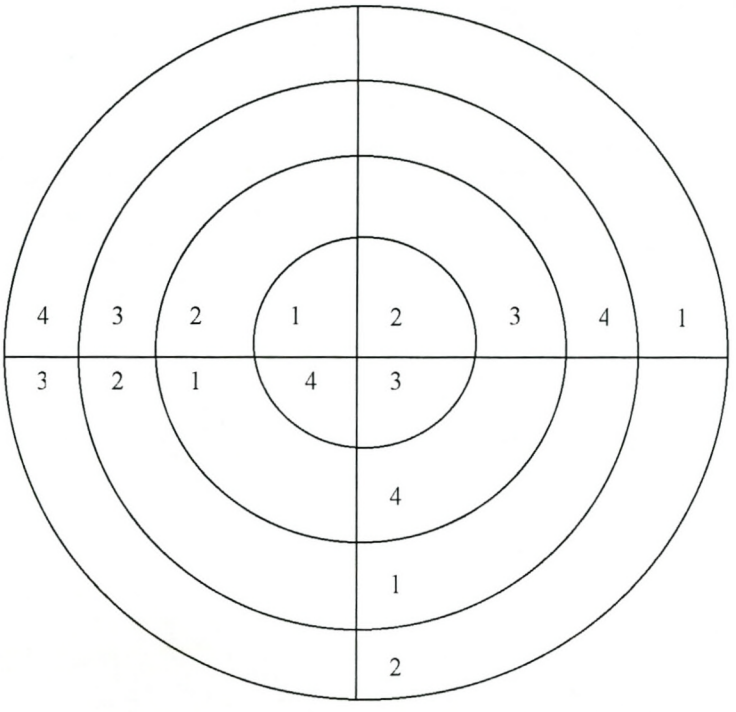


Figure 3.6. Layout of the disc that was used to ensure non-biased selection of dominant species.

CHAPTER 4. RESULTS AND DISCUSSION.

4.1. Strain selection.

Strain AB9 was selected because of its ability to produce large amounts of exopolymeric substances. It is widely accepted that EPS provide protection against antimicrobial compounds, including oxidizing biocides. Strain AB9 is a Gram-positive coccoid bacterium.

4.2. Effect of different ozone concentrations and exposure times on planktonic populations.

Strain AB9 showed sensitivity to O_3. In addition, O_3 concentration and exposure time had a notable effect on the number of surviving cells. Although no significant inhibitory effect was observed after exposure to the lower O_3 concentrations evaluated (Figs. 4.1 to 4.4), the higher O_3 concentration had an effect on cell viability following ≥7 min. exposure (Figs. 4.2 & 4.3). Reversible damage was observed in the case of lower O_3 concentrations (Fig. 4.4). There is no obvious explanation for this phenomenon. It may be possible that although there was still an O_3 residual remaining after exposure with the higher concentration (HCO) (thus prolonged exposure at inhibitory O_3 concentrations before sampling), the residual in the case of the lower O_3 concentration was too low to ensure sustained damage to the cells, because when ozone is added to water it has a half-life of 20 to 30 minutes (Baba *et al.*, 2002). Following the observation shown in Fig. 4.4 that a 15 min. exposure to the lower O_3 concentrations resulted in a temporary decrease in cell number, it was decided to increase the exposure time to 30 or 60 min. Similar to the shorter exposure times, there was no complete killing of the cells (Fig. 4.5), although a longer time (>5 h) was required for recovery. The total amount of O_3 added to the system during the 30 or 60 min. exposure was ~0.1 g and 0.2 g of ozone, respectively. To deliver the same amounts of O_3 to the system using HCO, exposure times of respectively 5.8 and 11.7 mins. would be required. This underscores the importance of considering concentration and exposure time when the objective is to completely kill all the cells.

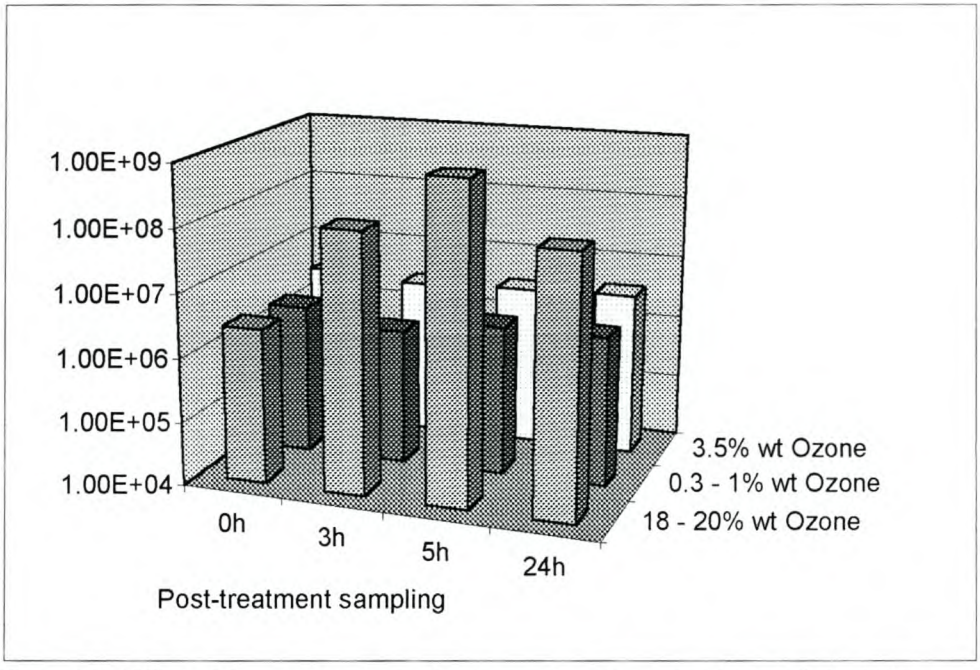


Figure 4.1. Effect of O_3 concentration and exposure time (5 min.) on bacterial survival.
LCO - denotes low concentration O_3.
HCO - denotes high concentration O_3.

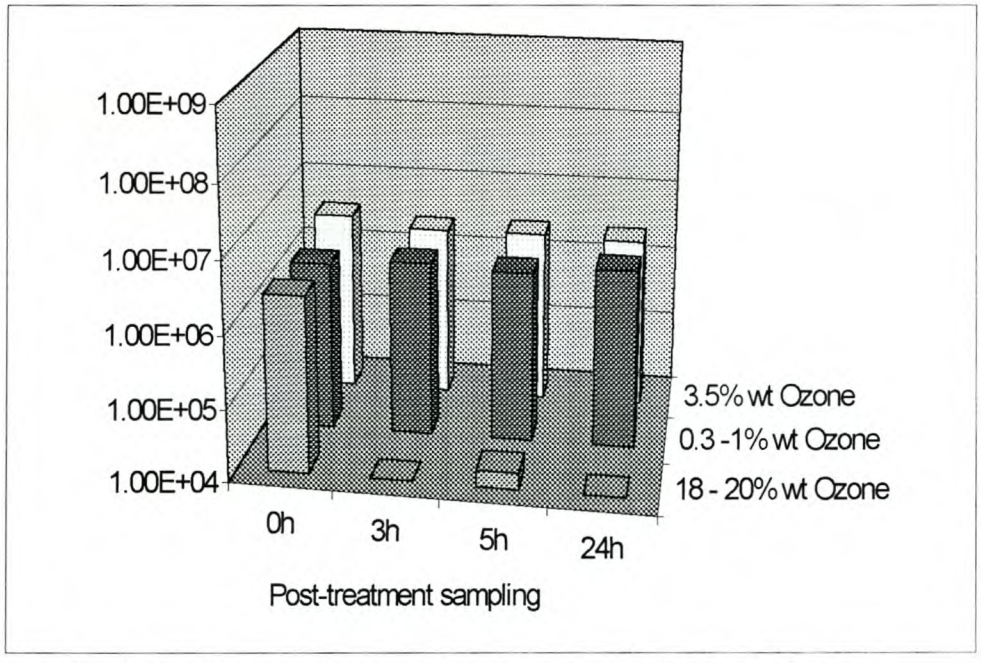


Figure 4.2. Effect of O_3 concentration and exposure time (7 min.) on bacterial survival.
LCO - denotes low concentration O_3.
HCO - denotes high concentration O_3.

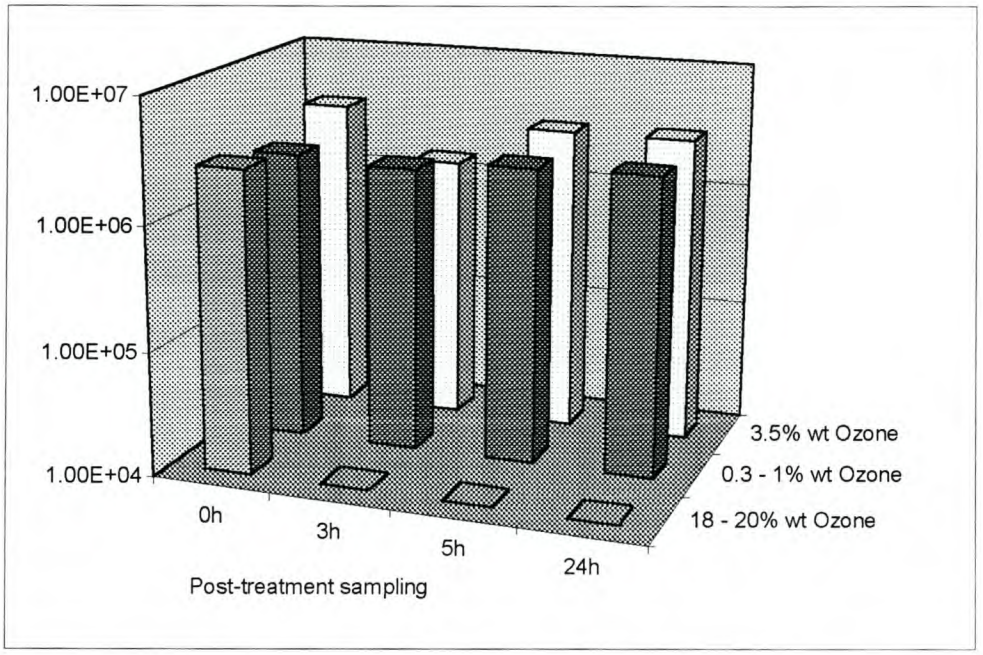


Figure 4.3. Effects of O_3 concentration and exposure time (10 min.) on bacterial survival.
LCO - denotes low concentration O_3.
HCO - denotes high concentration O_3.

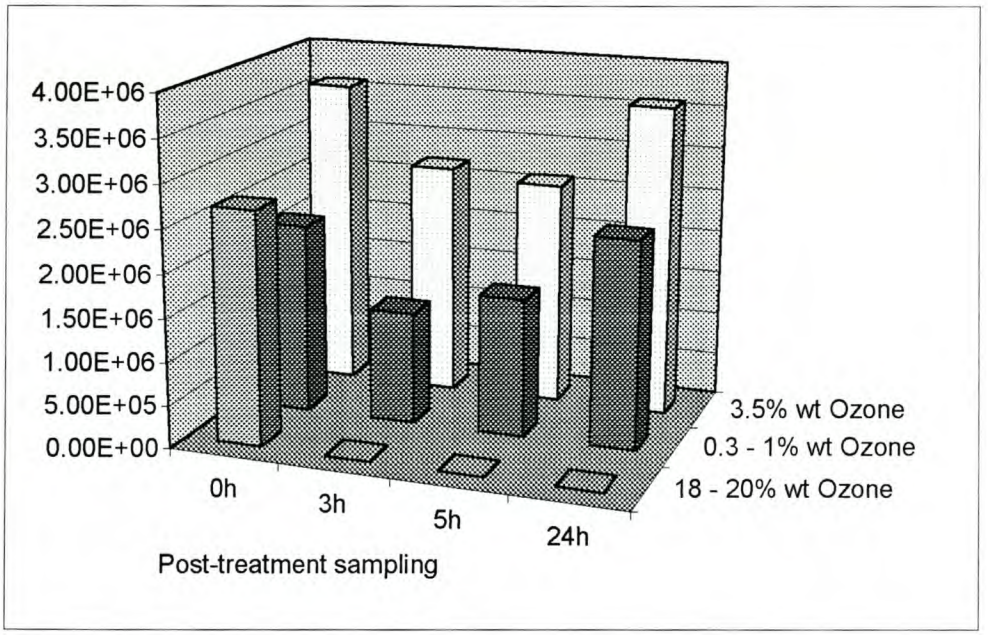


Figure 4.4. Effect of O_3 concentration and exposure time (15 min.) on bacterial survival.
LCO - denotes low concentration O_3.
HCO - denotes high concentration O_3.

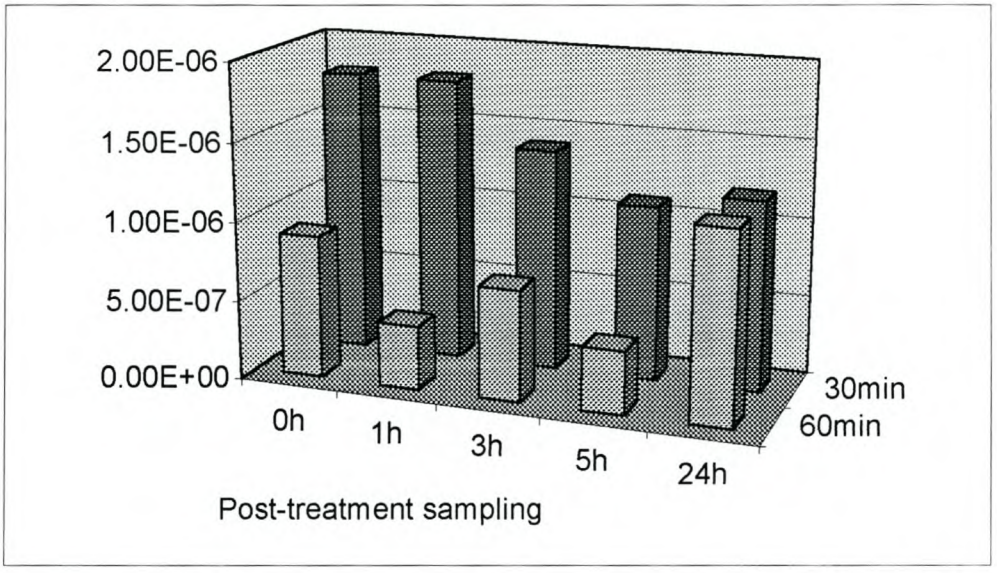


Figure 4.5. The effect of 3.5% wt O_3 over prolonged exposure times (30 min. & 60 min.) on bacterial survival.

4.3. Biofilm analysis.

4.3.1. Strain selection.

The characteristics of the six EPS-producing microorganisms that were used for these experiments are shown in Table 4.1.

Table 4.1. Organisms used for biofilm cultivation

Organism #	Gram reaction	Morphology
VJ1	-	**rod**
VJ2	-	**rod**
VJ3	+	**coccus**
VJ4	-	**rod**
VJ5	+	**coccus**
VJ6	+	**coccus**

4.3.2. Effect of different ozone concentrations and exposure times on biofilm integrity.

Previous studies done by Gard *et al.* (1992) showed that when organisms became attached in a biofilm, they were still present after 6 months. Geesey *et al.* (1978) showed that in industrial-, medical-, and dental areas of microbial ecology, the sessile surface cell count was higher than the planktonic populations by an order of 5×10^2 to 10^4. According to Highsmith *et al.* (1985) organisms growing in the planktonic state are more sensitive to biocidal attack than organisms growing in a biofilm.

In this study, exposure time had a notable effect on the number of surviving cells in biofilms. Fig. 4.6 shows typical data to illustrate the efficacy of O_3 to kill cells and to remove biofilm biomass from the surface. As expected, the higher ozone concentration exhibited a maximum inhibitory and dispersal effect especially after 1.5- to 3 hours of exposure, when compared to the untreated control (fig. 4.6), while the lower concentration O_3 had little or no effect (data not shown). When compared to the percentage of viable cells in the control (69 % of all cells in a field are viable), after 1.5- to 3 hours, the percentage of viable cells present was down to 37 % viable cells (fig 4.6).

When less dense biofilms were treated, a lower ozone concentration was required for the killing and removal of cells. Typical results are shown in fig. 4.7. When compared with the percentage of viable cells in the untreated control (95 % of all cells in a field are viable), after 2.5 hours exposure, 77 % of cells were viable. In addition to a reduction in the percentage of viable cells, cells were also removed from the surface. After exposure to 18% O_3, the total area coverage was reduced by 54%. In the case of 3.5% O_3, the reduction in the total area coverage was 59%.

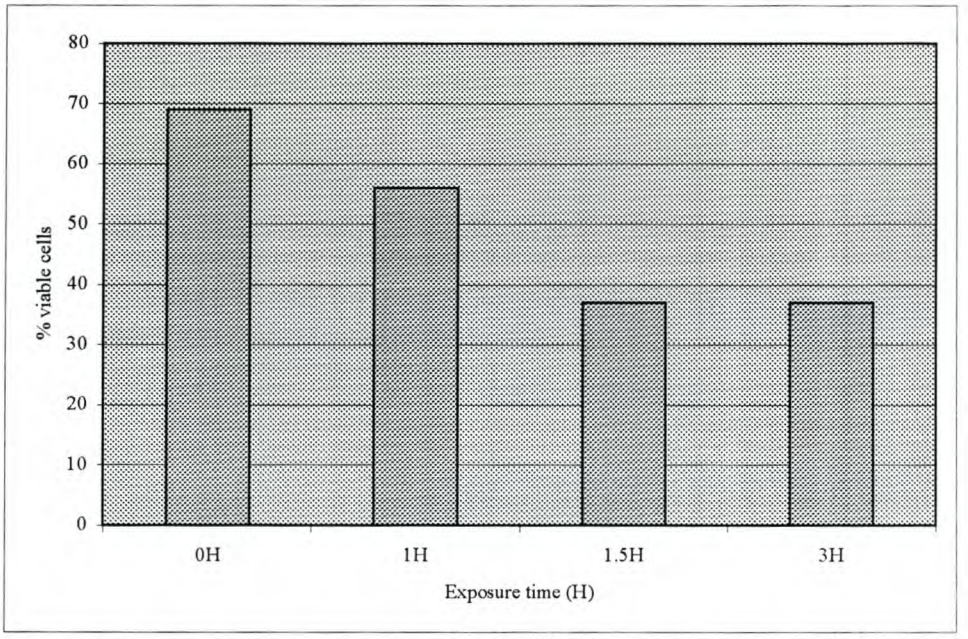


Figure 4.6. Effect of contact time on viability of biofilm cells and fouling (by thick biofilms) exposed to 18 wt %O_3.

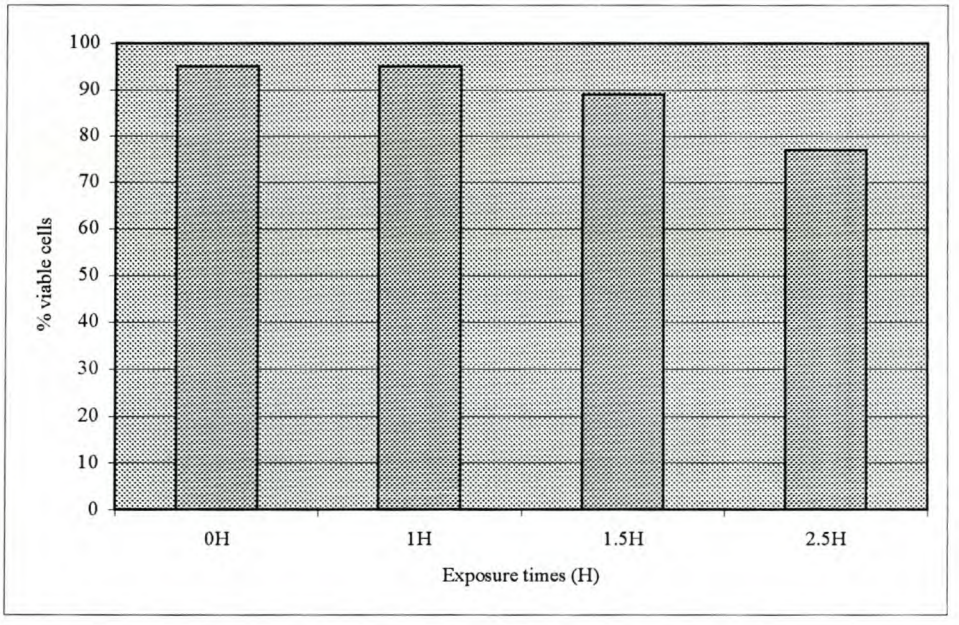


Figure 4.7. Effect of contact time on biofilms covering a relatively small area of the surface (less dense biofilm) exposed to 3.5% wt O_3.

4.4. Analysis using the batch culture technique.

4.4.1. Effect of HCO on biofilm integrity cultivated in a batch culture system.

There were no significant differences in the relative numbers of viable- and non-viable cells following treatment with HCO after exposure times of 0-, 5-, 7-, 10-, 15-, 25- and 60, minutes (fig. 4.8).

Fig. 4.9 shows the variability typically observed during these biofilm analyses when multiple experiments were performed. For instance, after 5 min. (45%), there was a reduction in the relative abundance of viable cells compared to the untreated control (50%), while after 7 minutes exposure there was an increase in the viable cell numbers to 65%. Such heterogeneity has been reported in the literature (e.g. Heydorn *et al.*, 2000).

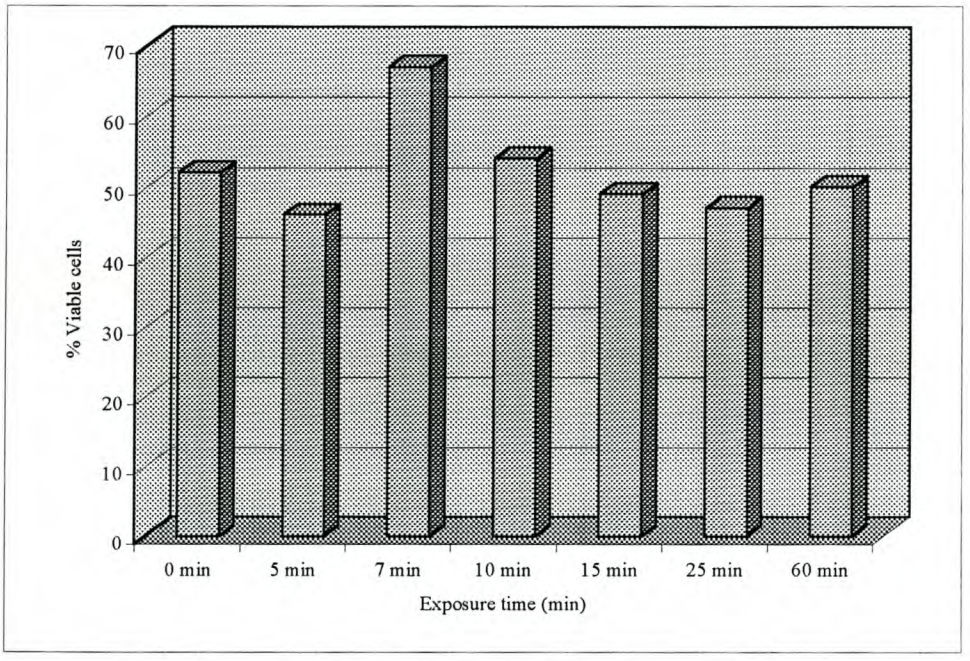


Figure 4.8. Number of viable cells as a percentage of total cells in the biofilm.

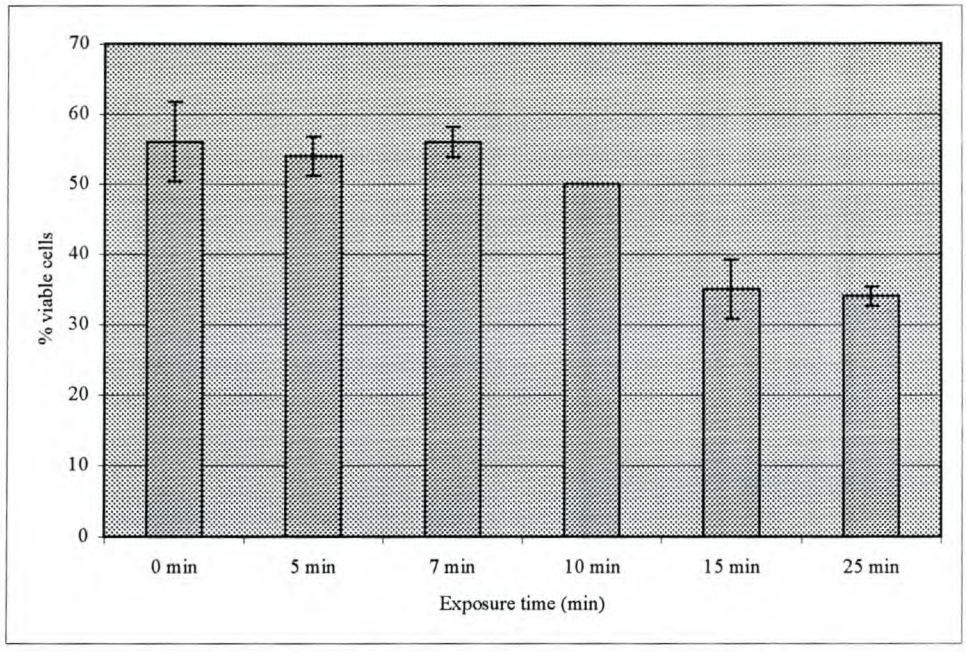


Figure 4.9. Data to show the highly heterogeneous nature of biofilms, and the subsequent high degree of variability as seen here by the varying length of the error bars.

4.4.2. Carbon utilization patterns by planktonic population cultivated in a batch culture system.

Figure 4.10 is a typical representation of the whole-community carbon source utilization patterns observed on the Biolog™ plates. From these results it can be deduced that there was a change in the community composition as a result of exposure to O_3. Compared to the community in the untreated control, which could utilize all the carbon sources, the surviving community after a 25 min. exposure to O_3, and the community after a recovery period of 3 hours, only a few of the carbon sources were utilized. However, thereafter, there was a gradual increase in the number of carbon sources that could be utilized, and the utilization profiles began to resemble that of the control (Fig. 4.11). It therefore seems plausible that there was not necessarily selective killing of certain species and a subsequent change in the community composition, but rather general reduction in the number of all members of the community followed by recovery in their numbers. For instance, there already was a marked recovery after 24 hours, as shown in Figs. 4.10 and 4.11. This could be due to the fact that the exposure time was not sufficient to kill all the cells of any of the respective community members, and who all subsequently showed regrowth. Regeneration of the surviving cells then meant that they were able to utilize

the carbon sources once more, hence the purple formazan colour change. This was then further demonstrated by the full utilization after 48 – to 168 hours (Fig. 4.11). Considering the reported half-life of O_3 in water with comparable carbon load than in these experiments of 25 to 30 minutes (Baba *et al.*, 2002) the residual ozone present in the media should not have an effect for a period exceeding these values. The results obtained from the biolog plates showed that regrowth must have occurred between 3- and 24 hours, where carbon utilization was beginning to resemble that of the control.

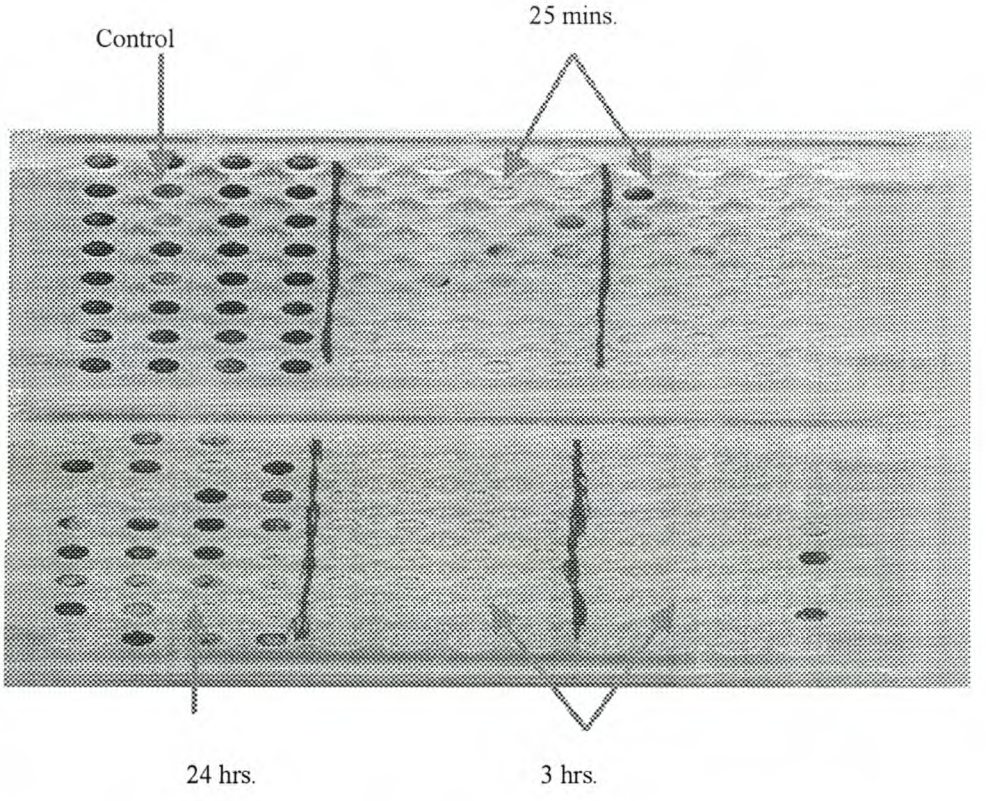


Figure 4.10. Typical example of a Biolog ™ plate to show the effect of O_3 on carbon utilization by a 6-member community. The community was exposed for 25 min. to 18% wt O_3. Sampling was performed before treatment, directly after the 25 min. treatment, and then at 3-, 24-, 48-, 96- and 168 h to determine whether there were changes in the species composition of the community as a result of recovery.

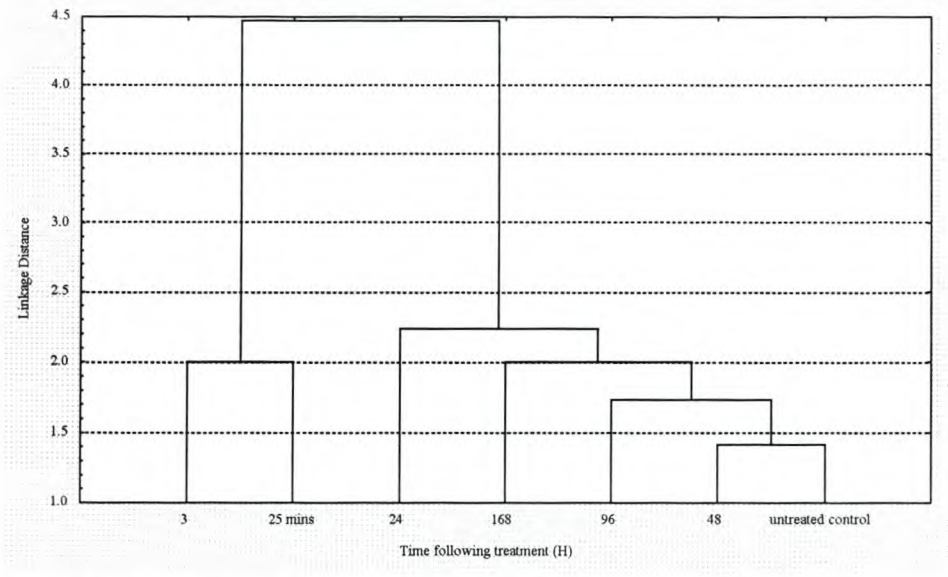


Figure 4.11. Effect of a 25 min. O_3 treatment on the carbon utilisation profiles of the planktonic community. These results demonstrated that, although there was a marked difference during the first few hours after treatment, the community profiles reverted to resemble that of the untreated control over time.

4.5. Effect of chlorine on carbon utilization patterns by biofilm communities.

The carbon utilization patterns of the chlorine treated samples were similar to that of the untreated samples (data not shown), suggesting that the treatment did not have a notable effect on the relative abundance of the individual community members. It was decided not to increase the chlorine concentration, because experiments with planktonic communities showed that this concentration was indeed sufficient to kill or inhibit a notable proportion of planktonic communities (data not shown). Giller *et al.* (1998) showed that the Biolog™ method was rather insensitive. These authors argued that such lack of sensitivity could be attributed to the fact that Biolog™ essentially reflects the activity of a wide range of organisms, with the ability to utilize a wide range of carbon sources. Furthermore, it was suggested that the activity of fast growing species could increase in the wells during the test (http://www.defra.gov.uk/research/Projects/Reports %5CPDF%5CSP0120.pdf) if fast growing species with the ability to utilize the majority of the carbon sources were present, they could potentially mask population shifts. Giller *et al.* (1998) also stated that when there is little stress, few microorganisms could dominate, allowing more microorganisms to be active, and affect carbon utilization patterns on the Biolog™ plate. The carbon utilization patterns in the exposed samples,

therefore, might not have been from the same organisms as in the untreated sample, i.e. if one species or group is lost, others will still metabolize the substrate. It could also be possible that the activity of growing species increased in the wells during the incubation time period (http://www.defra.gov.uk/research/Projects/Reports%5CPDF%5CSP0120.pdf). Because our aim with the experiment was to determine whether shifts occurred after treatment at concentrations relevant to those that has been used in other experiments, it was decided to evaluate another technique for comparison. T-RFLP analysis was selected for this purpose.

4.6. Microbial Diversity Analysis

4.6.1. Terminal restriction fragment length polymorphisms.

4.6.1.1. Whole-community profile analysis.

The difficulty in analyzing complex microbial communities is widely recognized. Especially to detect subtle changes in community composition. Marsh (1999) stated that the TRFLP technique had both high sensitivity and throughput, making it ideal for comparative analysis. TRFLP analysis was used in this study to determine whether the member composition of the treated community differed from the untreated community. The fluorescently labeled terminal restriction fragments (TRF) from *HhaI* (Liu *et al.*, 1997) digests revealed changes in the community composition associated with the absence or the addition of the biocide. The electropherograms for the untreated and chlorine-treated communities in Fig. 4.12 show the 3 PCR products digested with HhaI. Shifts from the untreated-, to the treated community could be observed by an increase in the presence of TRFs at 306 and 308 nucleotides at the 5' terminal end. In the untreated community, there was a peak at 175.88 nucleotides that was not present in the treated community. This indicated that the addition of the biocide to the system caused an alteration in the community composition, due to the disappearance of a peak that was clearly present in the untreated sample, but disappeared from the electropherograms of the duplicated treated sample. These results show that T-RFLP offers an approach to detect shifts in microbial community structure. In this instance, it was more sensitive than Biolog analysis. These results also suggest that, even though not detected with the other methods used, 4 mg/ml total chlorine had an impact on the biofilms. The longer-

term effect of this was not evaluated but could be an interesting topic for subsequent studies, especially in view of the potential of development of resistance against oxidizing biocides.

4.6.1.2. T-RFLP on individual community members.

Similar to observations made when the whole-community TRFs were evaluated, T-RFLP analysis of the dominant community members showed that the communities experienced a population shift in response to treatment. These shifts were not revealed by Biolog™ analysis. In comparison with the dominant species in the untreated control, electropherograms of individually selected colonies of the treated samples digested with HhaI yielded the results as shown in Fig. 4.13. For isolate S1, there was a disappearance of peaks at 162.54, 334.13 and at 335.71 nucleotides. Isolate S2 was similar to that of the control. There was, however a decrease in the area of TRFs at 162.54, 300.95, 334.17 and at 335.71 nucleotides. For isolate S6, there was a disappearance of peaks at 162.54, 334.13, and at 335.71 nucleotides. Similar to that of S1, S6 had a decrease in the area of TRFs at 298 nucleotides. Isolate S7 showed a disappearance of peaks at 162.54 and 298.78, and the appearance of peaks at 306.99 and 308.72, similar to that of isolate S3 and isolate S4 (Fig. 4.13). The area of TRFs at 306.99 and 308.72 nucleotides, increased in comparison to that of both isolate S3 and isolate S4 (Fig. 4.13). Avaniss-Aghajani *et al.* (1996) showed that 16S rDNA genes from phylogenetically related organisms result in the production of T-RF's of identical size. None of the samples contained the peak at 175.88 nucleotides that was present in the control, providing support for the contention above that there was indeed a shift in community composition as a result of treatment with chlorine at the concentration used.

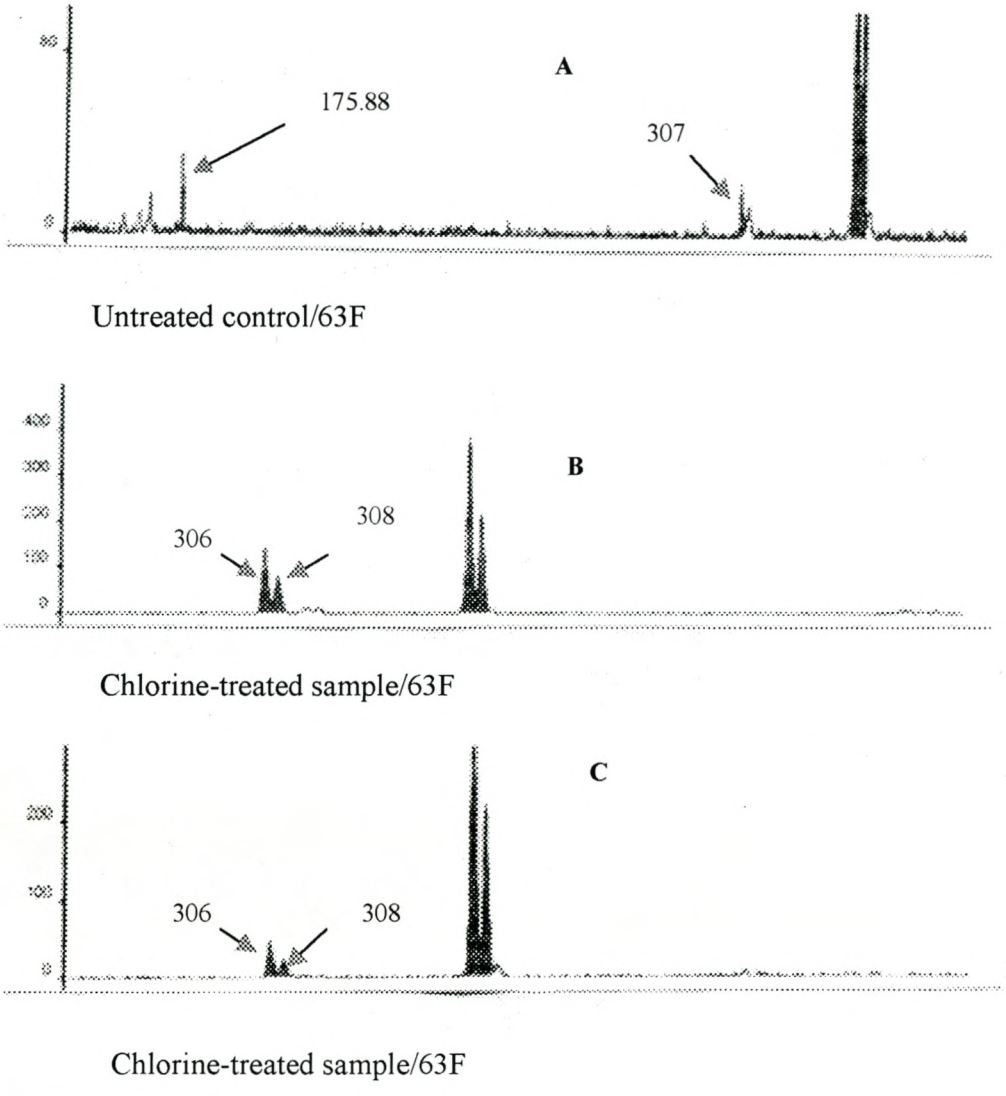


Figure 4.12. Examples of electropherograms (5' end) to show the difference in terminal restriction fragments between samples of the treated communities (B and C) and an untreated control (A).

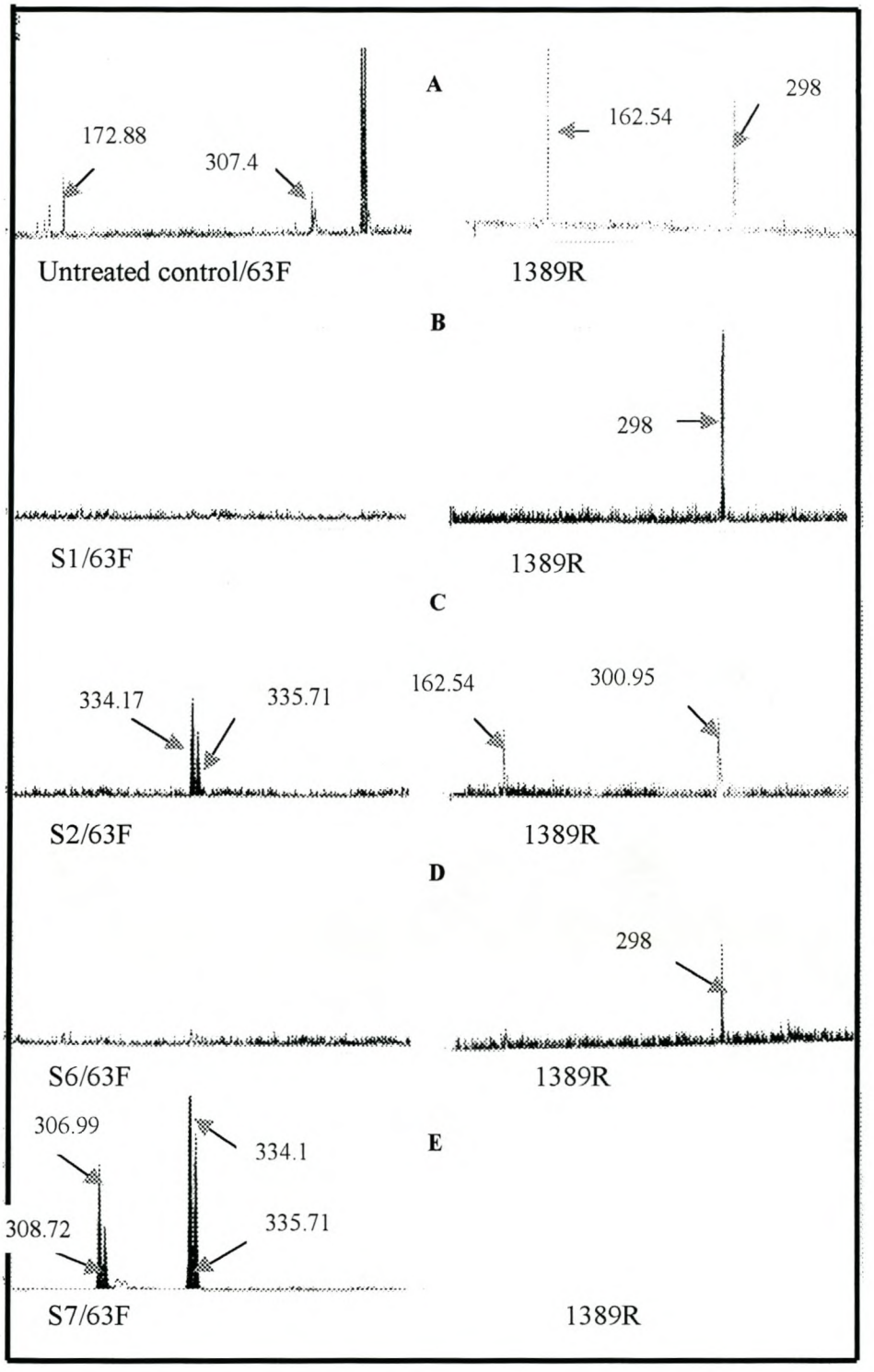


Figure 4.13. Examples of electropherograms (left –5'end; right 3'–end) to show the difference in terminal restriction fragments between isolates of the treated communities and untreated controls. In this case, only one organism (A) dominated in the untreated control. B – E represent the electropherograms of the community members that dominated following treatment.

4.7. General Conclusions

Similar to previous studies, organisms occurring in a biofilm were more resistant to oxidizing biocides than those growing in the planktonic state. These observations suggested that a contributing reason for resistance to biocide treatment might be due to the ability of microorganisms to attach and grow on non-sterile surfaces.

Contact time and the extent of biofilm development had an effect on the efficiency of O_3 in the killing of biofilm cells and the subsequent removal of biofilms from surfaces.

From the results obtained using the higher O_3 concentration, it was found that it effectively reduced microbial numbers and in doing so, effectively diminished the ability of the cells to recover. The results from these experiments suggest that although inherently more complex, utilization of the anodic oxidation of water to produce higher O_3 concentrations holds potentially promising benefits for industrial application.

Notable differences were observed between biofilms cultivated in batch and those cultivated in continuous culture. The biofilms grown in continuous culture were more rigid and generally more dense and extensive. For instance, the biofilms cultivated in batch were easier dislodged during preparation for microscopy, and showed increased sensitivity to lower biocide concentrations. These factors should be considered to ensure representative conditions when programs to control biofilms are being considered.

In the case of biofilm analysis, the Biolog™ technique proved to be less sensitive to reveal changes in community structure, shown by the similarity in the carbon utilization patterns, than T-RLFP.

TRFLP analysis showed that the addition of a biocide to a biofilm community does indeed alter the structure of the community with regard to dominant species, possibly selecting for more resistant populations.

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