THE USE OF BIODISPERSANTS AVAILABLE FOR BIOFOULING CONTROL IN INDUSTRIAL WATER SYSTEMS

Final Report to the
Water Research Commission

by

L Jacobs, E E de Bruyn and T E Cloete

Department of Microbiology and Plant Pathology
University of Pretoria
Pretoria 0002

WRC Project No. 592/1/97
ISBN 1 86845 276 X

July 1996

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EXECUTIVE SUMMARY

Water shortages force industries to recycle and to re-use water. This tends to concentrate dissolved and suspended substances in the water, stimulating microbial growth, biofilm formation and biofouling of industrial water systems. Programmes to prevent and control biofilm formation are therefore important to the relevant industries. To date, the monitoring of biofouling was complicated and time-consuming, involving culturing and counting of attached bacteria.

The overall aim of the project was to investigate the dispersing activity of the biodispersants available within the cooling water environment. This was addressed as follows:

Biodispersants were be investigated on a laboratory scale for two properties, i.e. prevention of bacterial attachment and detachment of established biofilms.

The envisaged products of the proposed research were the following:

- Quantitative information regarding the efficacy of biodispersants in the dispersion of biofilms in cooling water systems and
- training of qualified manpower with specialist knowledge regarding biofouling control.

A continuously circulating batch culture system was designed to study biofouling spectrophotometrically. Absorbance measurements of *Pseudomonas aeruginosa* attached to a glass tube was compared with direct counts of stained bacteria attached to 3CR12 steel coupons. Nonionic and anionic surfactants, the combined effect of a nonionic surfactant and a biocide, as well as the effect of an enzyme were evaluated for their efficacy in preventing and removing *Ps. aeruginosa* adhesion to solid surfaces.

Direct measurements of absorbance correlated well with the total counts using the staining technique. The former technique proved to be a reliable alternative to techniques requiring laborious counting of microorganisms. All the surfacants tested resulted in more than 90% inhibition of adhesion to the glass and metal surfaces. There was no significant difference in the efficacy of the different anionic surfactants as well as between the efficacy of the different nonionic surfactants. The nonionic and anionic surfactants resulted in more than 80% and 63% removal, respectively, of attached *Ps. aeruginosa* cells. There was no significant difference in the percentages of inhibition of bacterial attachment to solid surfaces using surfactants, when comparing the staining technique with spectrophotometric evaluations. The use of absorbance to determine the efficacy of a surfactant in preventing biofilm formation therefore proved to be a rapid and reliable method.

A nonionic surfactant used in combination with an isothiazolone biocide inhibited bacterial attachment by 95,5% and removed 97% of a mature *Ps. aeruginosa* biofilm. Separate use of the biocide and nonionic surfactant resulted in a 93,4% and 94,1% inhibition of bacterial attachment, respectively. An 88% and 91% removal, respectively, of the mature biofilm were achieved. Simultaneous use of the surfactant and biocide resulted in more efficient control of biofouling compared to the separate use of the biocide and surfactant.

The enzyme, evaluated in this study, displayed excellent anti-adhesive qualities, with 99% prevention of bacterial attachment to 3CR12 metal coupons and an 89% removal of a mature *Pseudomonas aeruginosa* biofilm.

Future work should include installing and using the flow-through system as an on-line monitoring system in the field for continuous monitoring of biofouling. Different combinations of surfactants and biocides need to be tested for biofouling control. More research also needs to be done on specific enzymes and their uses in biofouling control.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to the following persons and institutions who contributed to the success of this project:

* **The Water Research Commission**, for financial support;

* **Mr. Allan Hall**, Department of Microbiology and Plant Pathology, University of Pretoria, for his assistance with the Electron Microscope;

* The **WRC** Steering Committee members responsible for this project.

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CHAPTER 1

INTRODUCTION

Growth in population and expansion of industries result in an increasing demand on the water supplies of South Africa (Von Holy and Cloete, 1988). Water shortage forces the industries to recycle and reutilise water (Bondonno *et al.*, 1989). Recirculation of water concentrates dissolved and suspended substances (Cloete *et al.*, 1992). This stimulates microbial growth, biofilm formation and subsequently biofouling and macrofouling of industrial water systems (Cloete *et al.*, 1992). Biofilm formation results in increasing biomass deposition (Whitekettle, 1991) and fluid flow resistance, loss of heat exchange and microbially induced corrosion (Marshall, 1992) in water cooling systems, causing financial losses.

These adverse economic consequences of biofouling can be devastating for the industry (Lappin-Scott and Costerton, 1989). Biofilm development and programmes to prevent and control their formation are therefore important to the relevant industries. Attempts have been made to eliminate surface-colonizing of microorganisms using toxic compounds, such as chlorine and industrial microbiocides (Cloete *et al.*, 1992). This approach has only limited success. Although microbiocides are effective against planktonic microorganisms, considerable higher concentrations of these toxic materials are needed to inhibit the growth of these organisms when attached to surfaces (Characklis and Dydek, 1976). Increasing biocide concentration is costly, results in increasing environmental burdens and threatens non-target organisms (Whitekettle, 1991). Resistance to biocides has also been reported (Russel, 1990).

Surfactants have therefore become an important constituent of biocides (Cloete *et al.*, 1992). They are employed to achieve both uniform wetting of the surface to be treated and have an additional cleaning effect (Karsa, 1992). Inhibition of biofilm formation would result in most of the microorganisms existing in the planktonic state (Marshall, 1992). This can enhance biocide effectivity.

Traditional monitoring of biofouling rely on the culturing of the organisms after removal of the biofilm from metal studs from devices such as the Robbins device and the Pedersen device, or scanning electron microscopy investigations of the biofilm (Cloete *et al*, 1992). Counting bacteria by making use of culture medium cannot support the growth of the diversity of bacterial species involved in biofouling. Direct counting of DAPI stained cells gave more realistic and reliable results than culturing techniques (Wolfaardt *et al.*, 1991). However, staining and counting samples under the microscope is still a time consuming exercise. More recently, Image analysis and Confocal Laser Microscopy have been employed to give 3-D images of biofilms

(Caldwell and Lawrence, 1989). These methods are, however, highly sophisticated and not suitable for routine monitoring of biofilms. In light of the limitations of currently used monitoring techniques, a spectophotometric method was developed to overcome these disadvantages. The aim of this study was to develop a batch culture system to study adhesion of microorganisms to surfaces and to exploit this system for determining the efficacy of non-toxic surface active compounds as inhibitors of microbial adhesion as well as enhancers of biocide effectivity.

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CHAPTER 2

LITERATURE REVIEW

1. MICROBIAL BIOFILMS

1.1 WATER : THE HYDRO - ECOSPHERE

Two distinct populations are found in water systems, planktonic (free-floating) microorganisms present in the bulk fluid and the more predominant surface attached microorganisms growing in biofilms (Schapira, 1988). There are as many as 10 000 attached organisms present for each planktonic organism (Costerton and Lashen, 1984). Direct microscopic observations of microbial growth in diverse environments show that the majority of bacteria grow in enclosed biofilms attached to surfaces (Costerton and Lappin-Scott, 1989). Biofilms develop on virtually all surfaces immersed in natural aqueous environments, irrespective of whether the surface is biological (aquatic plants and animals) or abiological (stones, metal and concrete). Biofilms form rapidly in flowing systems where a regular nutrient supply is provided to the bacteria (Van Loosdrecht *et al.*,1990).

Extensive bacterial growth, accompanied by excretion of copious amounts of extracellular polymers, leads to the formation of visible slimy layers (biofilms) on solid surfaces (Marshall, 1992). Surface micro-environments are protective and offer resident bacteria a nutritional advantage (Costerton and Lappin-Scott, 1989). Many aquatic bacteria depend on attachment to surfaces in environments where nutrient concentrations are too low to allow growth (Heukelakiam and Heller, 1940). Biofilms capture inorganic and organic molecules from the bulk liquid, making it available as nutrients for the organisms in the biofilm (Costerton and Lappin-Scott, 1989). Cells within a biofilm are held closely together by the glycocalyx, ensuring an exchange of metabolites to sustain growth and allowing the removal of toxins (Costeron and Lappin-Scott, 1989). The combinations of enzymatic capabilities from several bacteria in consortium enable the degradation of a wide range of substrates (Costerton and Lappin-Scott, 1989). Glycocalyx formation protects biofilm microorganisms against antimicrobial agents, for example biocides and antibiotics (Costerton *et al.*, 1987).

Metabolites produced during bacterial growth lead to surface damage and biofouling. The consequences of surface biofilm formation for industries and medicine are detrimental and include the formation of reservoirs of potentional pathogens (Costerton *et al.*, 1987). Biofilms give rise to dental decay, metal corrosion, energy losses and material deterioration (Costerton and Lappin-

Scott, 1989). Biofilms may, on the other hand, be beneficial for example the production of fermented foods (Notermans *et al.*, 1991).

The fouling of ship hulls and water conduits by biofilms increases turbulence, which in turn increases fluid frictional resistance (Marshall, 1992). The resultant energy loss and reduced performance, along with the need for regular cleaning of ship or pipe surfaces, cost hundreds of millions of dollars per year on a worldwide scale (Costerton and Lappin-Scott, 1989). In addition, biofilms on surfaces of pipes and heat exchangers are responsible for financial losses to industry in the form of increased fluid flow resistance, loss of heat exchange and increased biomass deposition (Whitekettle, 1991). The attached microbial cells are also responsible for microbial induced corrosion (Ford and Mitchell, 1990).

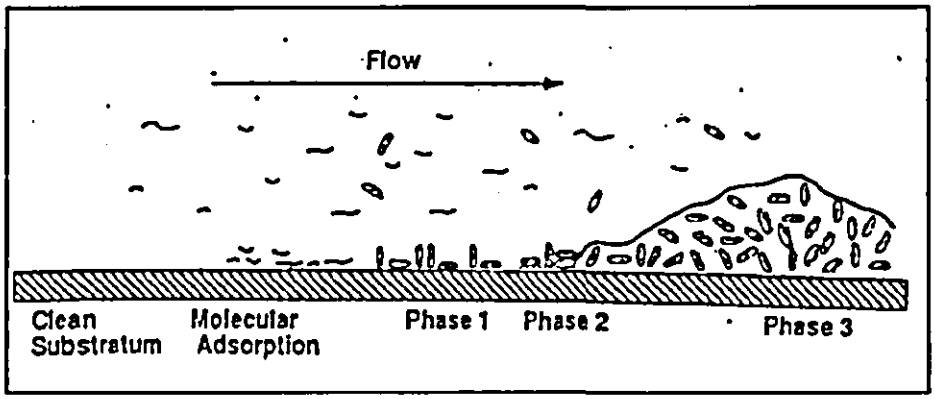
1.2 DEVELOPMENT OF BIOFILMS

Various models for biofilm formation have been proposed. Characklis and Cooksey (1983) and Characklis (1984) suggested that biofilm formation is a 5-stage process ie:

* Transport of nutrients, inorganic and organic matter to the solid surface.
* Absorption of a conditioning film to the surface containing inorganic or organic nutrients.
* Attachment of microbial cells to the wetted surface and initiation of growth.
* Bacterial metabolism within the biofilm.
* Cell disruption and detachment from the biofilm.

According to Lynch and Edyvean (1988) the development of biofilms occur in four stages ie:

* Immediately upon immersion, dissolved organic material is absorbed onto the surface to form a conditioning film.
* This is followed by the adhesion of microbial cells to the conditioned surface. Bacteria, diatoms and algae are carried to the substratum in association with suspended organic debris or mineral grain aggregates.
* Growth of the attached organisms occurs. These produce copious amounts of mucilage, resulting in the characteristic slime associated with biofilms. Growth continues until nutrient limitation occurs at the base, leading to the death of some organisms and a sloughing in parts of the biofilm.
* This is followed by re-establishment from the surrounding areas and the planktonic populations.



The various stages of biofilm formation as suggested by Marshall (1992) are shown in Figure 1.

Figure 1: Marshall (1992) suggested that biofilm formation starts with a freshly immersed substratum surface. Phase 1 represents initial colonization of the surface, phase 2 represents growth and extracellular polysaccharide production by the adhering bacteria and phase 3 the mature biofilm.

Wolfaardt (1990) listed the following parameters affecting the development of biofilms:

* System temperatures, which are related to season, day length, climate and wind velocity.
* Water flow rate past the surface.
* Nutrient availability.
* Roughness of the surface material.
* An approximately neutral pH of the water is optimal for the growth of most biofilm-forming bacteria.
* Particulate matter can become entrapped in the developing biofilm and provide additional attachment sites.
* Effectivity of biofouling control measures.

Exchange of substances only occurs on one side of the biofilm. Therefore, various gradients exist across the biofilm (Characklis, 1983). Respiration at the upper layer and fermentation in the middle layer with the release of products such as ethanol result in the formation of a nutrient gradient. An oxygen gradient also develops, as a consequence of bacterial respiration in the upper layer (Hamilton, 1987). When the biofilm has reached a thickness of 10 - 225 □m, conditions at its base are anaerobic (Hamilton, 1987). The biofilm now approaches a state of maturity, with a high species diversity and stability (Hamilton, 1987).

2. MICROBIAL ATTACHMENT

2.1 THE ROLE OF EXOPOLYSACCHARIDES (EPS) IN BACTERIAL ATTACHMENT

Adhesion to surfaces is a common and well-known behaviour of microorganisms in natural oligotrophic habitats (Zobell, 1943). Zobell (1943) suggested that, once bacteria adhere to a surface, firm attachment requires incubation for several hours. During this incubation period, extracellular adhesive materials are produced. Marshall and co-workers (1971) defined the two phases of sorption as follows: reversible sorption which is an essential instantaneous attraction of bacteria to surfaces. Bacteria are held weakly near the surface, exhibit Brownian motion and are readily removed by washing. Irreversible sorption involves the firm adhesion of bacteria to the surface, no Brownian motion is exhibited and they are not removed by washing.

Special cell surface structures (eg. fibrils or polymers) form strong links between cell and solid surface (Van Loosdrecht *et al.*, 1990). These polymers are essential for the development of surface films (Geesey, 1982). Davies *et al.* (1993) showed that the activation of the *algC* promoter in *Ps. aeruginosa*, is necessary for the production of exopolysaccharide alginate. This activation resulted from association of the bacteria with a Teflon substratum. However, Brown *et al.* (1977) presented evidence suggesting that excess polymer production may prevent adhesion.

The cells in the microcolony produce extracellular polysaccharides termed the glycocalyx (Costerton, *et al.*, 1981). Whitfield (1988) showed that bacterial extracellular polysaccharides occur in two basic forms. As a capsule (capsular polysaccharides) the polysaccharide is intimately associated with the cell surface and may be covalently bound to it. In contrast, slime polysaccharides are loosely associated with the cell surface. Fletcher and Floodgate (1973) found that primary acidic polysaccharides are responsible for the initial adhesion of bacteria. Secondary acidic polysaccharides predominant in preparations of attached bacteria, evolved from the primary polysaccharides after attachment. The secondary acidic polysaccharides, are fibrous, reticular substances stretched between and around adjacent bacteria.

2.1.1 CHEMICAL STRUCTURE OF EPS

Based on their chemical composition, antigenic specificity and mode of biosynthesis, the bacterial polysaccharides are naturally divided into two groups, namely specific and nonspecific polysaccharides (Christensen and Characklis, 1990).

a. Specific polysaccharides are called polysaccharide antigens and are specific

to individual bacterial strains. They are composed of linear or branched oligosaccharide repeating units. These oligosaccharide units are assembled into a polymer via lipid-linked intermediates.

b. Nonspecific polysaccharides are found in a variety of bacterial strains and are structurally different and generally simpler than the specific polysaccharides. They are homopolysaccharides, containing only one monomer.

Alginate is a linear copolymer of B - 1,4 - linked D-mannuronic acid and it's C -5 epimer, L-guluronic acid (Davies *et al.*, 1993). Heteropolysaccharides are generally composed of repeating structures and are mostly anionic (Wallace et al., 1994). Some components are unique to EPS, while others are also commonly found in other bacterial cell surface polysaccharides such as lipopolysaccharides and teichoic acid (Whitfield,1988). Neutral homopolysaccharides are also commonly found in bacteria (Whitfield, 1988).

2.1.2 FUNCTIONS OF BACTERIAL EPS

Most, but not all, of the functions ascribed to EPS are of a protective nature. The ability of a microorganism to surround itself in a highly hydrated EPS layer, may provide it with protection against desiccation and predation by protozoans (Whitfield, 1988). Cells inside a polymer matrix are inaccessible to antibacterial agents such as antibiotics (Costerton *et al.*, 1987). Anionic EPS may also bind and effect the penetration of both useful and toxic metal ions to the cell surface (Zottola, 1994).

Production of EPS, particularly in the form of capsules, is ordinary in pathogenic bacteria. The type of EPS, the amount synthesized and the rate of synthesis, may all have a bearing on the pathogenicity of an organism (Whitfield, 1988).

2.2 FACTORS AFFECTING BACTERIAL ADHESION

2.2.1 NUTRIENTS

An underlying belief in the literature is that nutrient limiting conditions enhance microbial attachment (Heukelakiam and Heller, 1940). Brown *et al.* (1977), demonstrated the growth of heterotrophic bacteria at the surface/water interface in carbon limited natural systems. According to Marshall (1992) some bacteria adhere more efficiently when starved, whereas others adhere well under nutrient-rich conditions. Marshall *et al.* (1971) showed that addition of glucose to a culture of bacteria growing in artificial sea water, inhibited attachment to glass. Kjellerberg *et al.* (1982) showed that a marine *Vibrio* which had attached due to low nutrient concentration regrew and left the surface upon increase in nutrient concentration. Bacteria growing in a glucose-limited medium would possess a maximum number of glucose receptor sites that would not be saturated and thus

promote attachment (Brown *et al.*, 1977). Glucose could on the other hand bind to aluminium as a molecular film and act as a bridge for the attachment of the bacteria through binding of the bacterial receptor sites to the glucose molecules (Brown *et al.*, 1977).

2.2.2 SURFACE TOPOGRAPHY

Attachment is dependant upon the chemical and physical interactions between the potential substratum and the organism (Fletcher and Loeb, 1979). *In-situ* experiments with a range of substrata have shown that attachment to high-energy (hydrophilic) surfaces such as glass, metals and metallic oxides increased with increasing exposure, whereas hydrophobic plastics such as polystyrene and plexiglass were colonized within a few hours of submersion (Pringle and Fletcher, 1983). Fletcher and Loeb (1979) found that large numbers of bacteria attached to hydrophobic plastics (Teflon and polystyrene) with little or no surface charge. Moderate bacterial numbers attached to hydrophilic metals (platinum) with a positive or neutral surface charge and very few attached to hydrophilic negatively charged substrata (glass and mica) (Fletcher, 1977).

2.2.3 AMOUNT OF SOIL ACCUMULATED

Dunsmore and Thomson (1981) found that the number of bacteria attaching at any time, was influenced by the amount of soil on the surface. The more soil accumulated, the more bacteria attached. The efficiency of a detergent was inversely related to the amount of soil on the surface.

2.2.4 SURFACE WETTABILITY

The wettability of the substratum (WA) is determined by contact angle measurement and expressed as the work of adhesion (Pringle and Fletcher, 1983). Pringle and Fletcher (1983) demonstrated the relationship between WA and the number of attached cells. The number of attached bacteria increased with WA to a maximum corresponding theoretically with a point at which the surface free energy of the substratum and the bacterial surface is equal.

2.2. 5 PROTEINS

To inhibit bacterial attachment, dissolved proteins must be able to affect at least one of the three components of the attachment mechanism, the bacterial surface, the attachment surface or the surrounding medium. Bovine serum albumin (BSA) prevents attachment through the absorption on, or reaction with, the bacterial extracellular adhesive material (Fletcher and Floodgate, 1973). BSA, gelatin, fibrinogen and pepsin inhibit bacterial adhesion through adsorption to the attachment surface (Fletcher, 1976).

2.2.6 CLEANING AND SANITIZING

Stone and Zottola (1985a) reported the effectivity of using two different recommended procedures when cleaning and sanitizing a milk pipeline. Proper detergent and sanitizer concentration as well as treatment temperatures inactivated *Ps. fragi* attachment and removed the biofilms. Sanitizers alone had little effect on the attached microorganisms. However, when the attached microorganisms were treated with cleaning compounds prior to treatment with sanitizers, the bacteria were inactivated (Krysinski *et al.*, 1992). Stone and Zottola (1985a) found that chemical concentration are responsible for destroying the attachment fibrils.

2.2.7 GROWTH PHASE

Fletcher (1977) reported that the highest amount of attachment occurred in the lag phase followed by a decrease in the number of cells attaching through the stationary and death phase. Weiss (1971) reported that 64% of *Ps. aeruginosa* cells in the log phase were piliated, but only 54% were in the stationary phase. Stone and Zottola (1985b) detected attachment of *Ps. fragi* within the first half hour of incubation, which represents a very early time in the lag phase. An increased log phase, culture age, cell concentration and time have an effect on the number of cells that attach and the attachment effectivity (Stone and Zottola, 1985b).

2.2.8 SHEAR STRESS

Stone and Zottola (1985b) showed that shear stress does affect initial attachment, with fewer numbers of microorganisms attaching as shear stress increases.

2.2.9 TEMPERATURE

Low temperatures of 3□C noticeably decreased the proportion of cells attaching when compared with attachment of cells at 20□C (Fletcher, 1977). Stanley and Rose (1967) showed that the strength of an adhesive polymer decreased with an increase in temperature.

2.2.10 CELL MOTILITY

Stanley (1983) found a 90% decrease in adherence when flagella were removed by blending. Their data indicated that loss of motility, rather than loss of attachment sites was the reason for the slower adherence of the blended cells.

2.2.11 pH

Attachment of *Ps. aeruginosa* to stainless steel was greatest in the pH range optimal for cell metabolism (Stanley, 1983). Stanley (1983) concluded that cell

attachment was assisted by the active transport of cations to the cell surface, increasing its surface charge.

2.3 BACTERIAL CELL WALL HYDROPHOBICITY

Cell surface hydrophobicity is an important factor in the adherence and subsequent proliferation of microorganisms on solid surfaces and at interfaces (Dahlbäck *et al.*,1981; Weiss *et al.*, 1982). Hydrophobic interactions play a role in the adherence of bacteria to wettable plastics (Rosenberg, 1981), phagocytes and the liquid hydrocarbon (Rosenberg *et al.*, 1980). Hydrophilic cells are expected to preferentially adhere to hydrophilic substrata while hydrophobic cells will adhere preferentially to hydrophobic substrata (Van der Mei *et al.*, 1993). Substratum and cell surfaces that are identically charged to one another will result in repulsive electrostatic interactions discouraging adhesion (Van der Mei *et al.*, 1993). Hydrophobic cells adhere to solid surfaces to a greater extent than hydrophilic cells (Van Loosdrecht *et al.*, 1987). At high growth rates, bacterial cells tend to become more hydrophobic (Van Loosdrecht *et al.*, 1987).

2.3.1 MEASURING CELL-SURFACE HYDROPHOBICITY

A number of methods for studying hydrophobic interactions of cells have been reported in the literature. These include binding of hydrocarbon and fatty acids to cells and cell components (Sharon *et al.*, 1986), measurement of the force required to remove hydrocarbon bound cells (Rosenberg *et al.*, 1980), partitioning of bacteria in aqueous polymer two-phase systems, hydrophobic interaction chromatography and contact angle measurements of dried cell layers (Rosenberg *et al.*, 1980).

Bacterial adherence to hydrocarbons is a simple, inexpensive and rapid method for investigating the hydrophobic surface properties of bacterial cells (Rosenberg *et al.*, 1980). The experimental approach is based on mixing washed cell suspensions with test hydrocarbons for a given time and measuring the adhesion simply as the decrease in turbidity in the aqueous phase after separation of the phases (Rosenberg and Doyle, 1990).

A few others have described bacterial adhesion in terms of surface free energy (Fletcher and Loeb, 1979; Absolom *et al.*, 1983; Busscher *et al.*, 1984). Surface free energy was calculated from the contact angle of a drop of water or another liquid on a given surface or on a closed layer of bacteria. Contact angle measurements (CAM) measure the surface free energies of solid surfaces (Van Oss, 1978). In addition to the contact angle method the hydrophobicity of bacteria can also be determined by partitioning bacteria between two aqueous phases or by quantifying the number of bacteria adhering to droplets of an organic solvent (Van Loosdrecht *et al.*, 1987). Hydrophobic interaction chromatography (HIC) measures microbial absorption to octyl - or phenyl - Sepharose beads (Rosenberg and Doyle, 1990). The salt aggregation test (SAT)

is a simple technique for studying the aggregative behaviour of cells in increasing concentrations of salting out agents (Rosenberg and Doyle, 1990). Rozgonyi *et al.* (1985) reported an improved SAT to sensitize the determination of cell-surface hydrophobicity. This technique is very rapid and sensitive. The two-phase partition measures the distribution of cells and cell components between two aqueous phases that are mutually immiscible (Rosenberg and Doyle, 1990).

Knowledge of hydrophobic surface properties and how they are mediated can facilitate manipulation of microorganisms, binding them to surfaces or removing them (Rosenberg and Doyle, 1990).

2.4 DETACHMENT

Detachment is defined as the transfer of biomass from the biofilm to the bulk liquid compartment (Characklis *et al.*, 1990). Allison *et al.* (1990) proposed the following mechanism for release from biofilm: Surface appendages associated with adhesion alter surface hydrophobicity. These structures are minimized during and immediately after cell division, leading to separation and dispersal of one daughter cell. Delaquis *et al.* (1989) reported an increase in hydrophobicity of *Ps. fluorescens* actively detaching from the biofilm.

A marine *Vibrio* which had attached due to low nutrient concentration regrew and left the surface upon increase in nutrient concentration (Kjelleberg *et al.*, 1982). However, Delaquis *et al.* (1989) showed that *Ps. fluorescens* detached from biofilm upon nutrient limitation.

In drinking water distribution systems, biofilm cells on the pipe walls detach, leading to increased planktonic cell numbers, which degrade the quality of the drinking water (Rosenberg and Doyle, 1990).

3. MONITORING OF BIOFILM FORMATION

3.1 MONITORING OF PLANKTONIC MICROORGANISMS IN WATER SYSTEMS

Historically, biofouling has been monitored by the quantification of planktonic microorganisms (Wolfaardt *et al*, 1991). However, attached microorganisms are predominant in aqueous environments and more than 10 000 attached bacteria for each planktonic cell have been reported (Geesey, 1982). This led to the shift in emphasis from planktonic bacterial monitoring to attached monitoring techniques (Cloete, *et al.*, 1992).

3.2 MONITORING OF ATTACHED MICROORGANISMS IN COOLING WATER SYSTEMS

Attached bacterial numbers exceed planktonic ones by 3 to 4 logarithm units in

water systems (Costerton *et al*, 1987). Yet the common practice of monitoring industrial water systems still involve the determination of planktonic bacterial numbers with the consequent underestimation of numbers and types of bacteria present in the biofilm. The full extend of bacterial numbers can only be determined by investigating microbial populations on the surfaces within the systems.

It is not always possible to obtained biofilm samples from within the systems. A number of devices have been designed for biofilm development such as the Robbins (McCoy et al, 1981) and Pedersen devices (Pedersen 1982).

3.2.1 *IN-SITU* DEPOSIT DEVELOPMENT PROBES

Test surfaces can be exposed *in situ* to pressurised injection water systems, using existing technology for access into the process pipework. Test surfaces are incorporated into holding assemblies compatible with the high pressure fittings so that sampling can be performed without partial shutdown and depressurisation of the system. The petrolite and Caproco bioprobes are the most common devices of this kind (Cloete *et al*, 1992).

3.2.2 SIDE-STREAM DEPOSIT DEVELOPING MONITORING DEVICES

Process water is taken from some point in the water injection system and pumped into a side-stream experimental rig incorporating the exposure surfaces, monitoring and control equipment (Cloete *et al*, 1992)

3.2.3.TUBULAR GEOMETRYBIOFILM MONITORING DEVICES

The tubular section most closely mimics the flow conditions encountered in water injection system pipeworks. The test liquid is pumped through the centre of the tube and the fouling deposits forms on the inside surfaces. The three types of devices used to monitor biofilms in this way, are:

* *Ported tubes*. This type of device consists of small diameter pipe fitted with a series of sampling ports containing removable studs that incorporate the test surface for exposure (Cloete *et al*, 1992).
* *Sectioned tubes*. The tubes may be presectioned and held within another tube assembly, or broken off as required or sectioned using a pipe cutter.
* *Monitored tubes*. This equipment measures the rate and extent of deposit growth by monitoring the effect of the accumulation on the heat transfer resistance (HTR) and fluid frictional resistance (FFR) (Cloete *et al*, 1992).

3.2.4. THE PEDERSEN DEVICE

The Pedersen Device carries replaceable sampling surfaces that allow more

detailed observation of relatively undisturbed portions of biofilm. Wolfaardt *et al*, (1991) used metal slides from a Pedersen device to follow biofilm development by staining attached cells with DAPI (4,6 - diamidino - z - phenylidole) and counting these cells by epifluorescence microscopy.

3.2.5 THE ROBBINS DEVICE

The Robbins device is a ported biofilm sampler consisting of removable test surfaces which are exposed to circulating fluids (McCoy *et al*, 1981). The Robbins device provides quantifiable samples of biofilms growing on submerged surfaces in aqueous systems. The stud surface of the device, which is exposed to the flowing bulk fluid, can be aseptically removed from the system and sampled for biofilm bacteria. The device can also be used to determine the concentration of biocides and antibiotics that kill planktonic bacteria in bulk fluids (Costerton and Lappin-Scott, 1989).

3.2.6 SCANNING CONFOCAL LASER MICROSCOPY

SCLM (Scanning Confocal Laser Microscopy) allows direct, nondestructive observation of biological materials (Lawrence *et al.*, 1991). The laser produces a high-intensity illumination, and since the returning signal is processed point-by-point, even low levels of fluorescence can be imaged with a sensitive photomultiplier (Wolfaardt *et al.*, 1994). This high sensitivity, and the capability to observe samples *in situ*, render SCLM suitable to demonstrate the presence and distribution of fluorescent molecules in biological material such as biofilms (Wolfaardt *et al.*,1994).

3.3 MONITORING OF BIOFILM THICKNESS

Biofouling can also be studied with scanning electron microscopy techniques (Brözel *et al.*, 1990), as well as Image analysis and Confocal Laser Microscopy, that give 3-D images of biofilms (Caldwell and Lawrence, 1989). These highly sophisticated methods are not suitable for routine monitoring of biofilms. In addition, methods such as the use of fluorescent stains cannot distinguish between living and dead cells (Gaylarde, 1990). Techniques involving the use of antibodies are sensitive and interferences may occur (Tatnall and Horacek, 1990).

The use of the total viable cell count technique is questionable, as only a fraction of the microorganisms present in the sample grow on a single medium (Cloete *et al.*, 1992). The enumeration of SRB (sulphate reducing bacteria) is particularly problematic due to their diverse requirements for both nutrients and environmental conditions (de Bruyn, 1993).

Gaylarde (1990) defined the requirements of rapid techniques for microbiological determinations as: (1) easy to use, (2) highly sensitive, (3) suitable for field use, (4) amenable to automation, (5) economic, (6) specific and (7) adaptable for use

with biocide treated samples. However, no single rapid technique has been identified that is able to meet all these requirements. Thus, due to the complexity and diversity of microbiological populations in cooling water systems, it is essential to use combinations in order to effectively and accurately assess the extent of microbiological growth or activity (Poulton, 1993).

4. BIOFOULING CONTROL

Since no plastic or metal surface has been shown to be resistant to bacterial colonization, control of biofouling is becoming an ever increasing study field. Biofilm formation can be controlled by reducing organic nutrients in bulk water (Costerton and Lappin-Scott, 1989). Industries control unwanted biofilms, with mixed success, using biocides or by incorporating toxic heavy metals into antifouling paints (Marshall, 1992). The use of biocides, especially chlorine, in water reticulation and heat exchange systems are successful only if the biofilm is removed manually, usually by pigging (forcing an expandable plug of some type through the pipe) after which the biocides are injected into the pipes at regular intervals (Marshall, 1992). Biofilms in heat exchangers can be removed by a patented process known as ice nucleation where the heat exchanger is frozen, and ice crystals physically dislodge the biofilm (Costerton, 1983). Dispersants and degrading enzymes can be used to disperse biofilms (Karsa, 1992).

4.1. BIOCIDES

The use of biocides to control biofouling in water cooling systems are an accepted practice (Cloete *et al.*, 1989). Although biocides are employed to reduce bacterial numbers, mere use of the correct biocide does not necessarily reduce the fouling rate, the correct dosage and frequency are essential (Cloete *et al.*, 1992).

Chlorination of a mature biofilm is usually unsuccessful because the biocide reacts only with the outer portion of the biofilm, leaving a healthy and substantial bacterial community on the surface that rapidly regrows (Marshall, 1992). Bacteria within a biofilm develop increasing resistance to biocides on repeated dosing. Repeated biocide applications tend to favour growth of bacteria producing copious amounts of extracellular polysaccharides (EPS) that protect the cells from the biocide (Sakagami *et al.*, 1989). Higher concentrations of toxic materials are then needed to inhibit the growth of attached organisms (Characklis and Dydek, 1976). Increasing biocide concentrations are costly, result in increased environmental burdens and threaten non-target organism (Whitekettle, 1991).

According to Cloete *et al.*, (1989), the building blocks of a successful biocide programme are:

* Knowledge of the organisms to be killed,
* Selection of the correct biocide or combinations and their respective

concentrations
* The dosage frequency,
* Monitoring the control of microorganisms,
* Monitoring microbiological attachment to surfaces.

RESISTANCE TO BIOCIDES

Few biocides, if any, are universal, and few are active against all bacteria (Cloete et al., 1992). Long term exposure of bacterial communities to sub optimum concentrations of the biocides can result in resistance. There are tree types of resistance documented:inherent resistance, also termed natural or intrinsic; acquired resistance due to mutation, and adapted resistance (Heinzel, 1988). Acquired resistance is usually plasmid encoded, the bacteria become resistant after acquiring the plasmid. An example is QAC (Quaternary ammonium compounds) resistance in *Staphylococcus aureus* encoded by a transposable element (Cloete *et al.*, 1992). Adair *et al.* (1969) reported certain pseudomonads growing on commercial QAC as nitrogen source because these contained ammonium acetate. The resistance of *Ps. aeruginosa* to benzalkonium chloride and chlorhexidine and that of *E. coli* to chloride dioxide is due to adaptation (Cloete *et al.*, 1992). In all these cases, resistance seemed to be due to increased EPS production. Adair *et al.* (1971) found that cells resistant to QAC had an increased lipid content of the cell envelope compared to the wild strains. QAC resistant cells contained more unsaturated fatty acids compared to wild strains (Jones *et al.*, 1989). Cloete and Brözel (1992) found that biocides induce cross-resistance to other biocides. The cross-resistance by induction indicates that a bacterial community can become more resistant to any given biocide after treatment with any other, than it had been before (Cloete *et al.*, 1992).

Biofilm bacteria are more resistant to biocides than the corresponding planktonic cells. Cloete *et al.* (1992) listed three reasons why biofilm bacteria are more resistant to antiseptics:

* Biofilms contain large amounts of EPS, which protects bacteria from biocides,
* Attached bacteria have a higher ratio of unsaturated to saturated fatty acids.
* Biofilm bacteria have a higher surface hydrophobicity due to attachment structures.

Bacteria respond to changes in their environment by profound phenotypic variations in enzymatic activity, cell wall composition and cell surface structure (Anwar *et al.*, 1985). These phenotypic changes involve the target molecules for biocides, antibiotics, antibodies and phagocytes as well as the external structures that control the access of these agents to the targets (Costerton *et al.*, 1987). The susceptibility to antibacterial agents is dictated by the structure of biofilms. Biofilms can increase the concentration of a soluble antibacterial agent in the

cellular environment by trapping and concentrating its molecules, as it traps and concentrates nutrients (Costerton *et al.*, 1987).

4.2 DISPERSANTS

Surfactants and emulsifiers are integral to many industrial, agricultural and food processors. Most of the compounds are chemically synthesized. Their surfactant and emulsification properties result from the presence of both hydrophilic and hydrophobic regions on the same molecule (Fiechter, 1992). Surfactants are an important constituent of biocides (Cloete *et al.*, 1992). They are employed to achieve both uniform wetting of the surface to be treated and have an additional cleaning effect (Cloete *et al.*, 1992).

Dispersants function by breaking up the foulants into smaller particles and keeping them suspended in cooling water (Strauss and Puckorius, 1984). This prevent deposit formation and enables foulant removal from the system via blowdown of filtration. Natural dispersants such as lignins and tannins provide good results but must be used continuously and at relative high dosages (50 to 200 ppm of active chemical) (Strauss and Puckorius, 1984). Unfortunately, these dispersants are derived from natural products, rendering them as nutrients and therefore making them excellent food for biological organisms and, when ingested, causing a loss in fouling control, increasing demand for microbiological deposits, and an increase in biological-control chemicals (Strauss and Puckorius, 1984). Additionally, these dispersants react rapidly with biocides, flocculants and other cationic chemicals.

Synthetic water-soluble polymers are the most common dispersant chemicals currently used. Synthetic dispersants represent a major improvement over natural dispersants for several reasons: they can be made to any specific molecular weight; they are not easily degraded by biological organisms; they do not react with chlorine or iron salts; and, most important they cost less for the same performance (Strauss and Puckorius, 1984).

Surfactants are also commonly used for oily or gelatinous foulants. They are low foaming, nonionic surfactants that are added at dosages of 10-20ppm (active). Surfactants damage the cell by reducing its permeability: they disrupt the normal flow of nutrients into the cell and the discharge of wastes, causing the organism's death (Cloete *et al.*, 1992). Anionic surfactants reduce cell permeability and eventually dissolve the entire membrane (Strauss and Puckorius, 1984). There is a correlation between the efficacy of surfactants and their ability to lower the surface tension of the aqueous environment (Whitekettle, 1991). The surfactants capable of reducing the surfaces tension by □20 mNm^{-1} showed the greatest efficacy as inhibitors of microbial adhesion (Table 1) (Whitekettle, 1991).

Table 1. Efficacy of surface-active compounds against bioadhesion to steel. (Whitekettle, 1991)

Compound	Efficacy rating	Surface tension reduction (mNm^{-1})
Emulsen	0	0.0
Disodium ethylene diaminotetraacetate (EDTA)	0	0.2
Polyacrylic acid	0	0.8
Sodium lignosulfonate	0	1.5
Poly (maleic anhydride) co-diiso-butylene	0	5.4
Sodium lauryl sulfate	1	7.0
Polyvinyl alcohol	1	13.0
Methylcellulose	1	13.0
Polyethylene glycol ether of secondary alcohol	1	20.6
DP-1254A	2	20.8
DP-1254B	2	27.2
Phosphate ester	2	20.4
DP-1253	2	23.7
DP-1252	3	20.0
DP-1255	3	25.0
DP-1223	3	29.0
Alkyl, dimethyl benzyl ammonium chloride	3	26.0

4.2.1 ANIONIC SURFACTANTS

The hydrophilic group of an anionic surfactant is an anion, of which the most common anions are sulfonates and sulfates (Attwood and Florence, 1983). Alkyl aryl sulfonates are the most widely used surfactants, since they have excellent detersive power, are made of easily available materials which make them low in price and their formulations have attractive properties. They consist of alkyl chains, with a mixture of 10 - 15 C atoms, but principally C_{11} and C_{12} are attached to the benzene ring in the para position to the sulfonate group (Attwood and Florence, 1983). These surfactants can be used as its sodium salt as the sole surfactant or in conjunction with other anionic, non-ionic or cationic surfactants (Karsa, 1992). The surfactant concentrates in the water surface of aqueous solutions to lower the surface tension.

At low concentrations ($1 mgL^{-1}$) surfactants dissolves in water normally and each individual molecule or ion is present as a separate entity. As more is added, a concentration is reached ($100\text{-}1000 mgL^{-1}$), depending on the salt content of the solution, the temperature and the chemical nature and structure of the surfactant, at which micelles are formed. This concentration is called the critical micelle concentration (CMC). Micelles contain molecules which are orientated with their hydrophobic groups clustered together and the hydrophilic ends extending outwards. Beyond the CMC much more surfactant can be dissolved and the micelles increase in number. Micelles play a part in the cleaning action of

surfactants (Attwood and Florence, 1983).

4.2.2 NON-IONIC SURFACTANTS

Surface active agents that have no electronic charge ere referred to as non-ionic surfactants (Karsa, 1992). Non-ionic surfactants have the advantage over ionic surfactants in that they are compatible with all other types of surfactants and their properties are generally little affected by pH (Attwood and Florence, 1983). The non polar regions are mainly derived from hydrocarbon, alcohol or fatty acid sources with carbon skeletons in the range of C_8 - C_{20} (Attwood and Florence, 1983). The polar region is usually provided by a polyoxethylene glycol. Examples of non-ionic surfactants are: n-octyl glucoside, Triton and polyethylene oxide (10) cetyl ether.

4.2.3 QUATERNARY AMMONIUM COMPOUNDS

Quaternary ammonium compounds (QAC) adsorb onto the cell membrane and chemically react with the negative charge associated with the cell wall (Cloete *et al.*, 1992). These cationic surface-active chemicals are organically substituted nitrogen compounds that ere generally more effective against algae and bacteria in alkaline pH ranges (Strauss and Puckorius, 1984). Their action is attributed to their positive charge, which forms an electrostatic bond with negatively charged sites on microorganism cell walls (Strauss and Puckorius, 1984). The electrostatic bonds create stresses in the wall, leading to cell lysis and death. The QAC also cause cell death by protein denaturation; cell-wall permeability is distorted, reducing the normal intake of life-sustaining nutrients to the cell (Strauss and Puckorius, 1984). Problems associated with the use of these compounds are that they lose their activity in heavily fouled systems. Because of their surface activity, they tent to emulsify oils instead of participating in cell-wall bonding (Strauss and Puckorius, 1984). These compounds become less effective as temperature increases. Anionic surfactants promote the inactivation of QAC's (Cloete *et al.*,1992).

4.3 ENZYMES

Certain enzymes are available which break down the polysaccharide matrix of the glycocalyx, thus breaking down the substance which attaches bacteria to surfaces and each other (EDCI Programme. Economics Laboratories, St. Paul, MN) (Cloete *et al.*,1992). The break down of the EPS matrix leaves the cells exposed, and therefore accessible to other surfactants and biocides. Without the polysaccharide matrix, cells are dispersed into the bulk liquid where it can be disposed of by dispersants or other chemical compounds used to kill bacteria in cooling water. Using enzymes may prevent the formation of a polysaccharide matrix, thus preventing the formation of biofilms and biofouling. It is, however, still a new concept to use enzymes in the control of biofouling in industrial water systems and more research is needed in this specific field.

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CHAPTER 3

SPECTROPHOTOMETRIC MONITORING OF *Pseudomonas aeruginosa* ADHESION TO A GLASS SURFACE

INTRODUCTION

Microorganisms attach to surfaces in order to survive (Cloete *et al.*, 1992). This was first suggested by Heukelakiam and Heller (1940) as well as Zobell (1943) who found that bacteria can grow more readily, at low organic nutrient concentration, after attachment to available surfaces, presumably because adsorbed nutrients are concentrated and thus more accessible. This attachment leads to the development of microbial biofilms (Costerton and Lappin-Scott, 1989), causing serious fouling problems in many aquatic systems. In industrial water systems biofouling may cause serious financial losses by inducing metal corrosion and resulting in energy losses (Costerton and Lappin-Scott, 1989). The monitoring of biofilm formation has therefore become a well-studied field (McCoy *et al.*, 1981; Eigmy *et al.*, 1983; Bakke and Olsson, 1986; Challinor, 1991). However, methods currently in use to detect and quantify biofouling in industrial water systems have severe limitations.

In recent years the monitoring of biofouling shifted from the determination of planktonic bacterial numbers to that of sessile bacterial counts (Cloete *et al.*, 1992). This was mostly because planktonic bacterial numbers may underestimate or overestimate the extent of the problem.

Devices such as the Robbins device (McCoy *et al.*, 1981) and the Pedersen device (Pedersen, 1982) have proven useful in the study of undisturbed portions of biofilm.

Unfortunately these techniques rely on the culturing of the organisms after removal of the biofilm from the metal studs, or scanning electron microscopy (SEM) investigations of the biofilm (Brözel, 1990; Costerton *et al.*, 1987). Counting bacteria by making use of culture media is not only time-consuming, but the composition of a single culture medium cannot support the growth of the diversity of bacterial species involved in biofouling. A lot of expertise and sophisticated equipment are needed to obtain results with SEM techniques, as well as Image analysis and Confocal Laser Microscopy, that give 3-D images of biofilms (Coldwell and Lawrence, 1989). These highly sophisticated methods are not suitable for routine monitoring of biofilms.

More recently, Wolfaardt *et al.* (1991) described the use of DAPI (4,6 - diamidino - z - phenylidole) to quantify sessile bacteria. Direct counting of DAPI stained cells gave a more realistic and reliable estimations of biofouling than culturing techniques. The method is also

relatively easy to perform. Although the use of DAPI offers a number of advantages over conventional culturing techniques, it also has limitations. The samples must be stained and counted under the microscope. This is a time-consuming exercise. Results are therefore not immediately available. After treatment the samples cannot be used for further studies.

The major drawback of currently used techniques to study biofouling is that bacteria numbers must be determined, whether on culture plates or under the microscope. In this study, we developed a spectrophotometric method to overcome these disadvantages. By using absorbance to determine biofouling we eliminated the need for culturing and counting of bacteria.

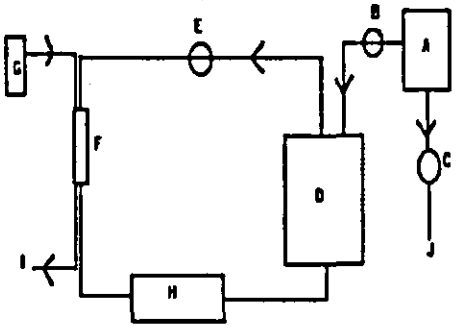

Figure 1. Diagram of the Continuous flow.

Through system with (A) the sterile medium, (B), (C) and (E) peristaltic pumps, (D) the reservoir, (F) the flow through glass tube, (G) the sterile distilled water, (H) the modified Pedersen device, and (I) and (J) the effluent.

This method was compared to the quantification of the attached cells by DAPI-staining and scanning electron microscopy.

MATERIALS AND METHODS

Organism used. A wild strain of *Pseudomonas aeruginosa* isolated from a cooling water system and identified in a previous studywas used (Brözel and Cloete, 1992) .

Continuous flow - through system. A modified Pedersen device (McCoy *et al.*, 1981) and a flow-through tube were connected in series with a peristaltic pump, which in turn was connected to a 4 l reservoir (Fig. 1). *Pseudomonas aeruginosa* was cultured in 200 ml R2A broth (Reasoner and Geldreich, 1985) for 24 h at room temperature. Of this culture 20 ml were used to inoculate the reservoir containing 4 l R2A medium. The flow rate through the system was 1,8 ml/h. Sterile medium was continuously fed into the reservoir at a dilution rate of 240 mℓ/h. The same amount of used medium and cells left the reservoir, keeping the cells in the same growth phase at all times and ensuring a constant concentration of 10^8 cells/ml in the reservoir and the flow-through system.

The modified Pederson device. The modified Pederson device was used because it allows the study of a relatively large surface area of undisturbed biofilm (Fig. 2). The following additional modifications were made: (1) the device was perspex, instead of steel coupons, (2) the size of microscope slides were used instead of microscope cover slips, (3) only one test pile was used, and (4) coupons were vertically installed instead of horizontally. Twenty 3CR12 metal coupons were held in place in the device. Coupons, 75 x 27 x 1mm in size were used for the DAPI-staining. Smaller coupons (25 x 27 x 1mm) were used for the scanning electron microscopy studies. Before each experiment, the coupons were prepared as described by Wolfaardt *et al.* (1991). Prior to use, the device was cleaned with a soap solution, thoroughly rinsed, sterilized with 5% hydrogen peroxide for 10 min. and flushed with sterile distilled water to remove any residues.

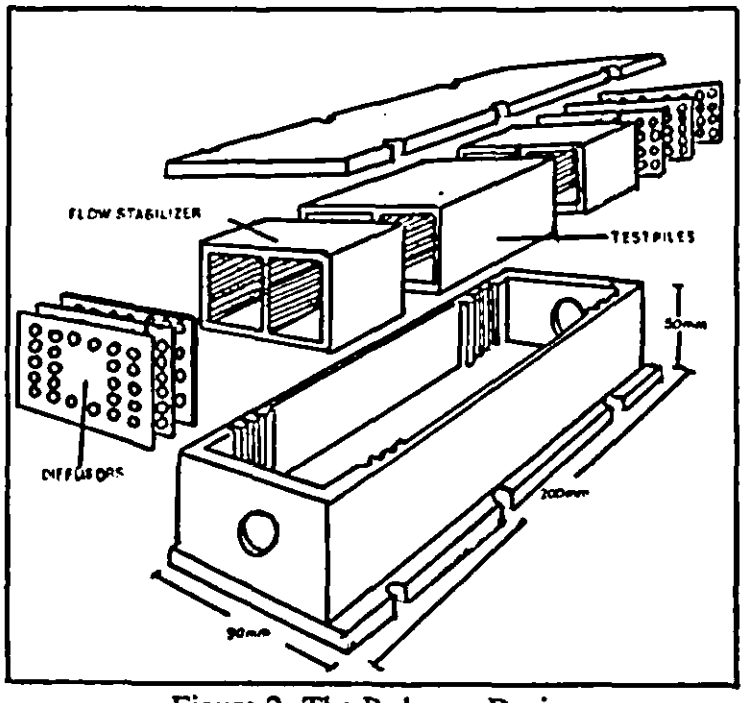

Figure 2. The Pedersen Device.

The flow-trough glass tube. A flow-through glass tube was constructed for the spectrophotometric analysis (Fig. 3). Before each experiment the tube was washed with a soap solution (Extran), rinsed with distilled water and dry sterilized in an oven for 6 h at 180□C.

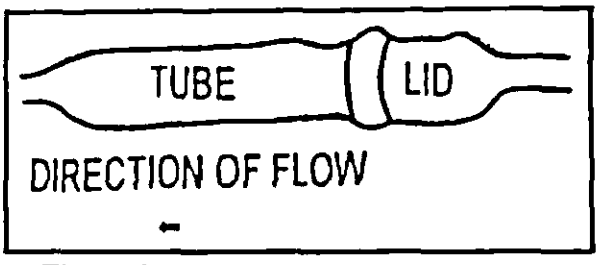

Figure 3. The Flow - through glass tube.

Scanning Electron Microscopy (SEM). Coupons (25 x 27 x 1mm) were removed, in duplicate, from the modified Pedersen device at 4, 8, 24, 28, 32, 48 and 56 h, with a sterile forceps and replaced with a sterile coupon, in order to keep the flow constant. After removal the coupons were rinsed with sterile distilled water for 30 s to remove any unattached cells and then fixed for SEM by the following series of treatments: 2% gluteraldehyde (1 hour); 0.175M Phosphate- buffer (3 x 15min.); 50% ethanol (1 x 15min.) ; 70% ethanol (1 x 15min.); 90% ethanol (1 x 15min.) and 100% ethanol (3 x 15min.). The coupons were thereafter dried in a critical point dryer, mounted on studs and coated with gold plasma and examined using the Hitachi S-450 scanning electron microscope.

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

Spectrophotometric measurements. An absorbance measurement of the bacteria attached to the glass tube was done simultaneously to the removal of the coupons from the modified Pedersen device. The SQ 118 Spectrophotometer (Merck, PTY.LTD), adjusted to a wavelength of 550nm, was used. Before each measurement, a zero adjustment was done on the spectrophotometer using a clean tube filled with distilled water. The absorbance of the bacteria attached to the flow-through tube was determined after carefully rinsing and replacing the medium in the tube with sterile distilled water.

Statistical analysis. Linear regression analysis was performed on the results obtained in the quantification of biofilm formation over time, using the DAPI staining technique and direct spectrophotometric monitoring. Correlation coefficients were calculated for the different data sets.

RESULTS

Ps. aeruginosa started to adhere to the coupons within the first half hour of exposure to the bacteria (Fig. 4 and 5), which is in accordance with the results of Stone and Zottola (1985). An increase in numbers is visible, up to the stage where the entire surface of the coupon is overgrown. The formation of micro colonies occurred after 24 h of exposure (Fig. 4c and 5d) and brought along the layering of cells, which resulted in the formation of a layered biofilm after 48 h (Fig. 4d and 5f). The production of extracellular polysaccharides (EPS) could be seen 24 h after exposure (Fig. 4C). After 56 h a levelling off of the adhesion rate took place. The absorbance measurements of attached bacteria cells to the glass tube, increased within the first 4 h after initiation of the experiment (Fig. 6). After 8 h the adhesion of bacteria on the glass surface could be seen with the naked eye.

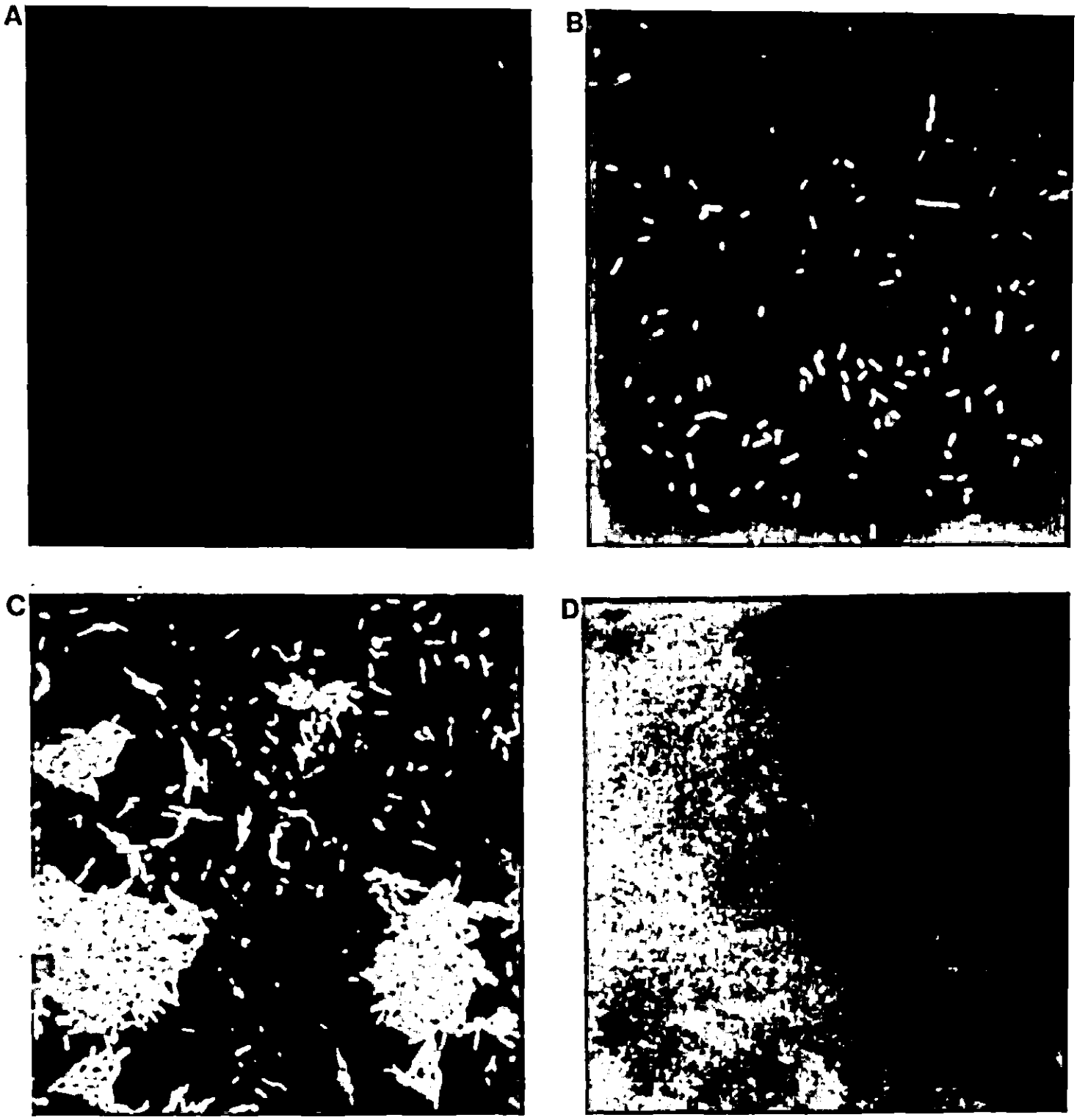

Figure. 4. DAPI-stained bacteria attached to 3CR12 coupons, after (A) 4 h, (B) 8 h, (C) 24 h, with micro colony formation and (D) attachment after 48 h, with bacteria adhering in layers to form a biofilm (800 X magnification).

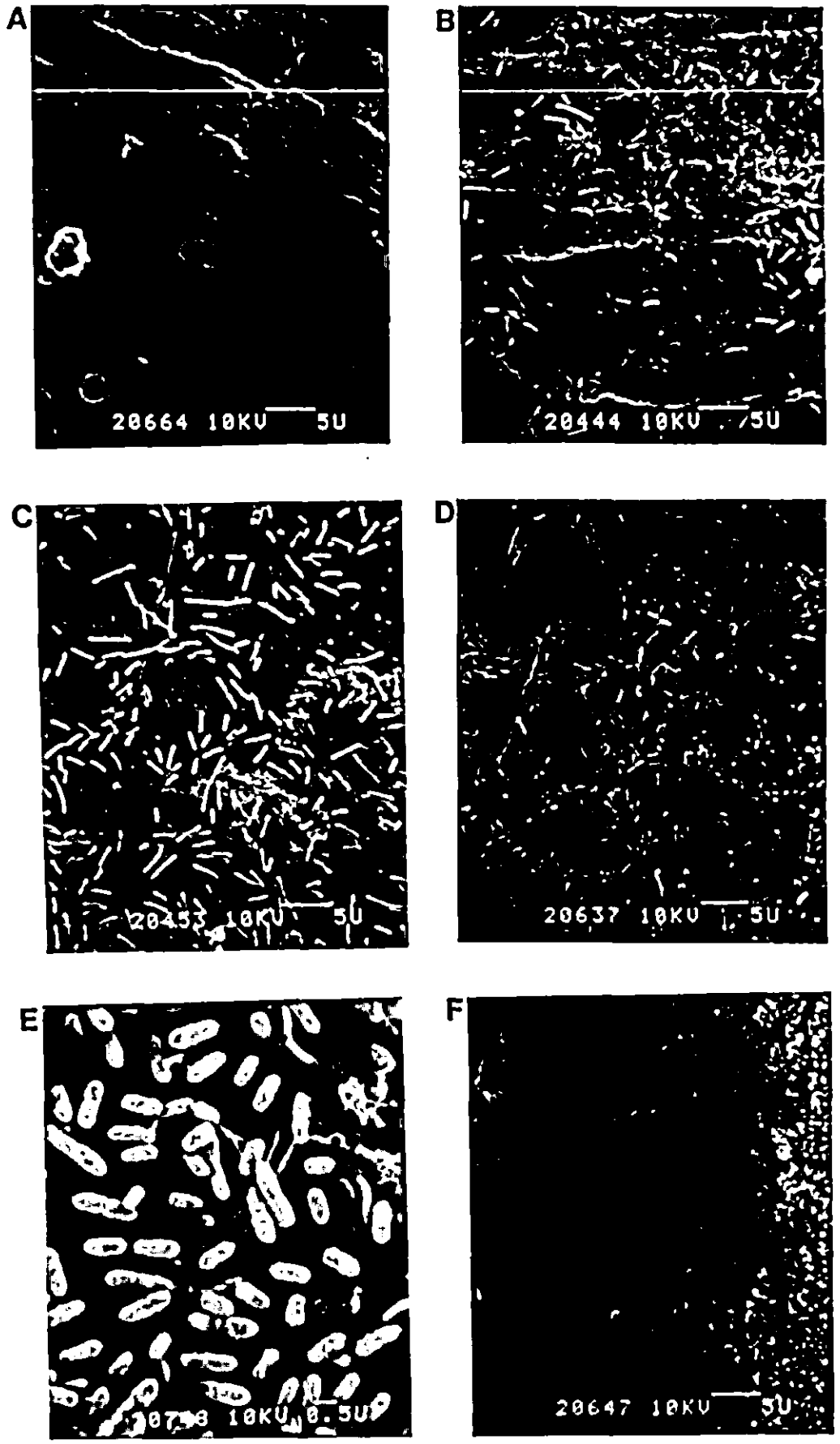

Figure. 5. SEM photomicrographs of attached *Ps. aeruginosa:* (A) the control with no bacterial adhesion, (B) the attachment after 4 h, (C) after 8 h, (D) 24 h, (E) 24 h with EPS production and (F) after 48 h. The bars represent 5 □m for A, B, C, D and F and for E the bar represents 0,5 □m.

The attachment rates of *Ps. aeruginosa* to the Pedersen device metal coupons as determined by DAPI total counts and to the glass tube surface as determined by direct absorbance measurements are shown in Figure 6. There was a linear relationship between the DAPI technique and time (r^2 = 0,944) as well as absorbance measurements and time (r^2 = 0,927) in determining biofilm formation.

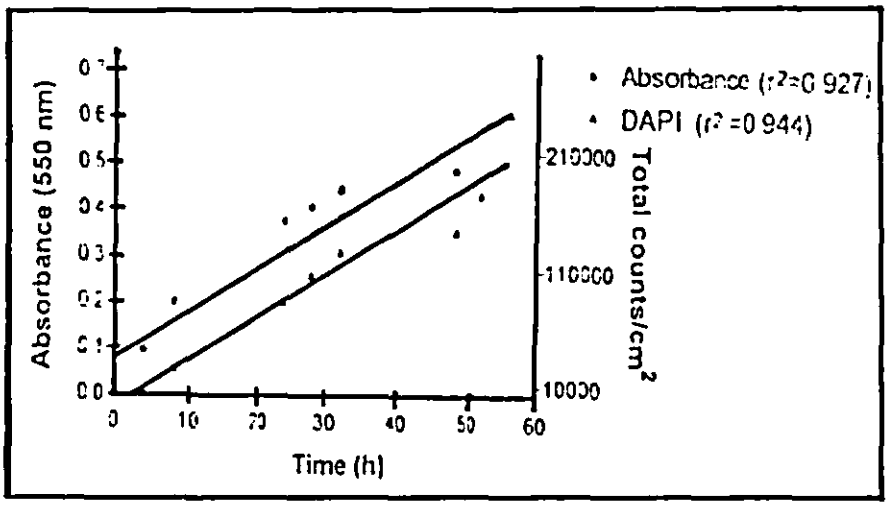

Figure. 6. Linear regression analysis of direct bacterial counts using the DAPI-technique and that of the absorbance measurements of attached *Ps. aeruginosa* cells (550nm) over a period of 56 h.

A linear relationship between the DAPI technique and direct absorbance measurements (r^2 = 0,925) for biofilm formation monitoring was also demonstrated (Fig.7).

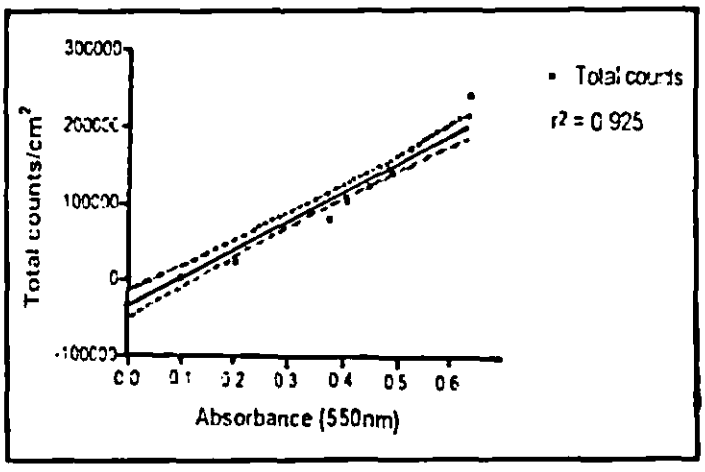

Figure. 7. Linear regression correlation between the DAPI-technique (total counts/cm^2) and the absorbance (550 nm) of the attached *Ps. aeruginosa* cells.

DISCUSSION

The DAPI technique and absorbance measurements , used in this study to quantify biofouling, correlated in a linear fashion with time (Fig.6). The direct measurement of absorbance also correlated well with the total counts obtained using the DAPI technique (r^2 =0,925). Figure 7 demonstrates the linear relationship between the two techniques. Both these techniques therefore proved to be reliable for monitoring biofilm formation.

However measuring absorbance in a glass tube is uncomplicated and an inexpensive method for obtaining reproducible results. Visual evaluation was also possible since the increase of attached bacteria could be seen with the naked eye. The advantages of using absorbance to measure the rate of colonization are: (1) results are immediately available for evaluation, which means that an immediate forecast can be done on the seriousness of biofouling, (2) no staining, counting or culturing needs to be done, (3) the glass tube can be used in more than one test, and (4) the biofilm can be used for further studies, such as specie diversity and identification of attached bacteria. Another application for this technique would be the evaluation of biocide and biodispersant efficacy for the control of biofouling in the laboratory. The flow-through system can also possibly be installed and used as an online monitoring system in the field, for continuous monitoring of biofouling. This application, however, requires further investigation.

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CHAPTER 4

METHODS FOR THE EVALUATION OF ANIONIC AND NONIONIC SURFACE-ACTIVE COMPOUNDS FOR THE PREVENTION AND REMOVAL OF *Pseudomonas aeruginosa* ADHESION TO SOLID SURFACES

INTRODUCTION

Adhesion to surfaces is a common and well-known behaviour of microorganisms in oligotrophic habitats (Zobell, 1943). Bacteria colonize surfaces to survive in nature because nutrients are more available at the solid-liquid interface (Lawrence *et al.*, 1987). This adhesion and subsequent metabolism lead to the formation of biofilms (McCoy *et al.*, 1981). Bacterial biofilms promote increasing biomass deposition (Whitekettle, 1991), resulting in fluid flow resistance, loss of heat exchange and microbial induced corrosion (Marshall, 1992).

Industries control unwanted biofilms, with varying degrees of success, using biocides or by incorporating toxic metals into antifouling paints (Marshall, 1992). The use of biocides, especially chlorine, in water reticulation and heat exchange systems is effective only if the biofilm is removed manually. Chlorination of a mature biofilm is usually unsuccessful because the biocide only reacts with the outer portion of the biofilm, leaving a healthy and substantial bacterial community on the surface that rapidly regrows (Marshall, 1992). Bacteria within biofilms develop increasing resistance to the biocide on repeated dosing (Cloete *et al.*, 1992). Brözel and Cloete (1992) found that biocides also induced cross-resistance to other biocides.

More recently surface active compounds (surfactants) have been employed to prevent bacterial adhesion to surfaces. Surfactants have also been incorporated as an important constituent of biocides (Cloete *et al.*, 1992). Surfactants result in both uniform wetting of the surface to be treated and have an additional cleaning effect (Cloete *et al.*, 1992). Whitekettle (1991) found a correlation between the ability of a surface-active compound to lower surface tension and its ability to prevent microbial adhesion. According to Paul and Jeffrey (1985), dilute surfactants completely inhibited the attachment of estuarine and marine bacteria. Brij 56 (polyethylene oxide (10) cetyl ether) totally inhibited the adhesion of *Pseudomonas* sp. NCNB2021 to hydrophobic polystyrene, but had little effect on adhesion to hydrophilic glass (Humphries *et al.*, 1986). It is unlikely that surfactants will have any mutagenic effects on bacteria, or that microorganisms would be able to become resistant to the action of surfactants, as can be the case with biocides (Russel, 1990; Brözel and Cloete, 1992). Although biocides are one of the alternate treatments for control of biofilm formation in industrial water systems (Cloete *et al.*, 1992) little published information is available on the effectivity of different biodispersants against bacterial attachment.

The aim of this study was to compare the efficacy of various anionic and nonionic surfactants

to prevent bacterial adhesion to both steel and glass surfaces and to remove a mature biofilm from a steel surface. In a previous study, we developed a spectrophotometric monitoring method for *Pseudomonas aeruginosa* adhesion to a glass surface (see chapter 3). The use of spectrophotometry was therefore also evaluated as a laboratory method to estimate the efficacy of these surfactants to inhibit biofilm formation, or to remove an existing biofilm.

METHODS AND MATERIALS

Organism used.
Pseudomonas aeruginosa isolated from a cooling water system and identified in a previous study (Brözel and Cloete, 1992) was used for all the experiments.

Surfactants used.
A range of nonionic and anionic surfactants were obtained from the major South African suppliers (Table 1). The dosing concentration of the surfactants was 20 ppm according to manufacturer instructions.

Evaluation of surfactants.
The compounds were evaluated for their efficacy in preventing *Ps. aeruginosa* adhesion to the 3CR12 coupons as well as removal of a preformed biofilm on the coupons. The nonionic and anionic surfactants were tested separately at the appropriate concentrations. *Ps. aeruginosa* was cultured in 200 ml R2A broth (Reasoner and Geldreich, 1985) for 24 h at room temperature. Of this culture, 20 ml were used to inoculate the reservoir containing 4 ℓ of R2A broth. The surfactant was introduced to the reservoir at the beginning of the experiment for determining its ability to prevent bacterial adhesion to solid surfaces. In determining the surfactants' ability to remove pre-attached bacteria, a biofilm was formed by allowing the bacteria to adhere to the surface of the 3CR12 coupons for 168h, before the dosing of the surfactants was started. During both the experiments, the surfactants were fed continuously into the system. A control system, where no surfactants were added throughout the duration of the experiments, was also included.

The continuous flow - through system and modified Pedersen device used for the experiments are described in chapter 3. Biofilm formation was monitored using Scanning Electron Microscopy (SEM), the 4,6-diamidino-2-phenylidole (DAPI) staining technique and spectrophotometric measurements (chapter 3).

Expression and statistical analysis of results:
The results are expressed as the mean value ($\pm$) of the standard error means for each treatment. Levels of statistical significance were calculated using the students t-test.

RESULTS

Adhesion of Ps. aeruginosa to 3CR12 coupons and a glass tube in the absence of surfactants:
Ps. aeruginosa readily adhered to the 3CR12 coupons and the glass tube in the absence of the surfactants (control system). The number of bacteria adhering to the surfaces increased to $1{,}24 \times 10^5$ bacteria / cm^2 after 32h as determined using the DAPI-staining technique (Fig.1).

Absorbance measurements also demonstrated increased attachment of *Ps. aeruginosa* to the glass tube over time in the control system (Fig. 2).

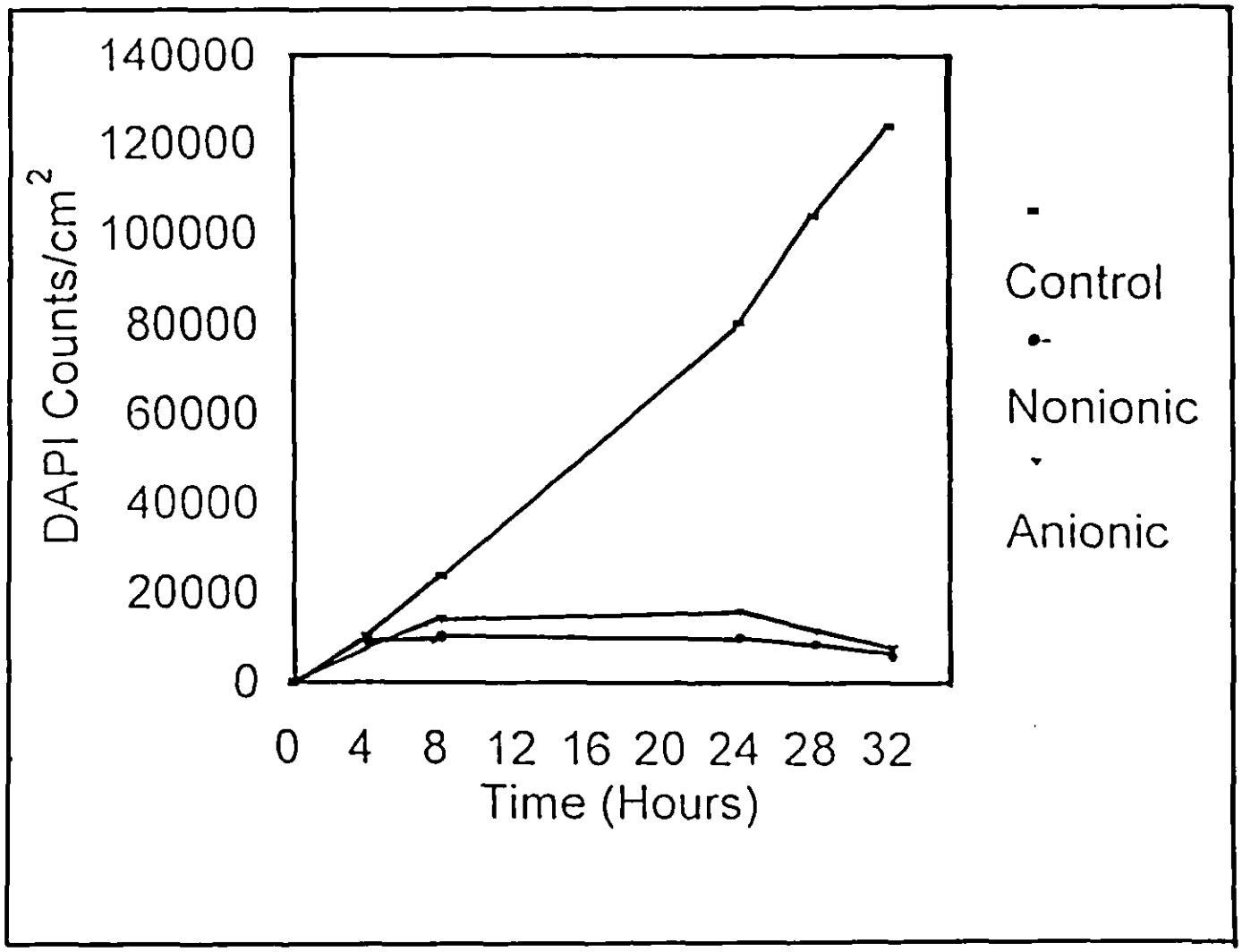

Figure 1 Number of DAPI-stained *Ps. aeruginosa* cells adhering to the 3CR12 coupons over a period of 32h in the presence of 20 ppm of surfactant

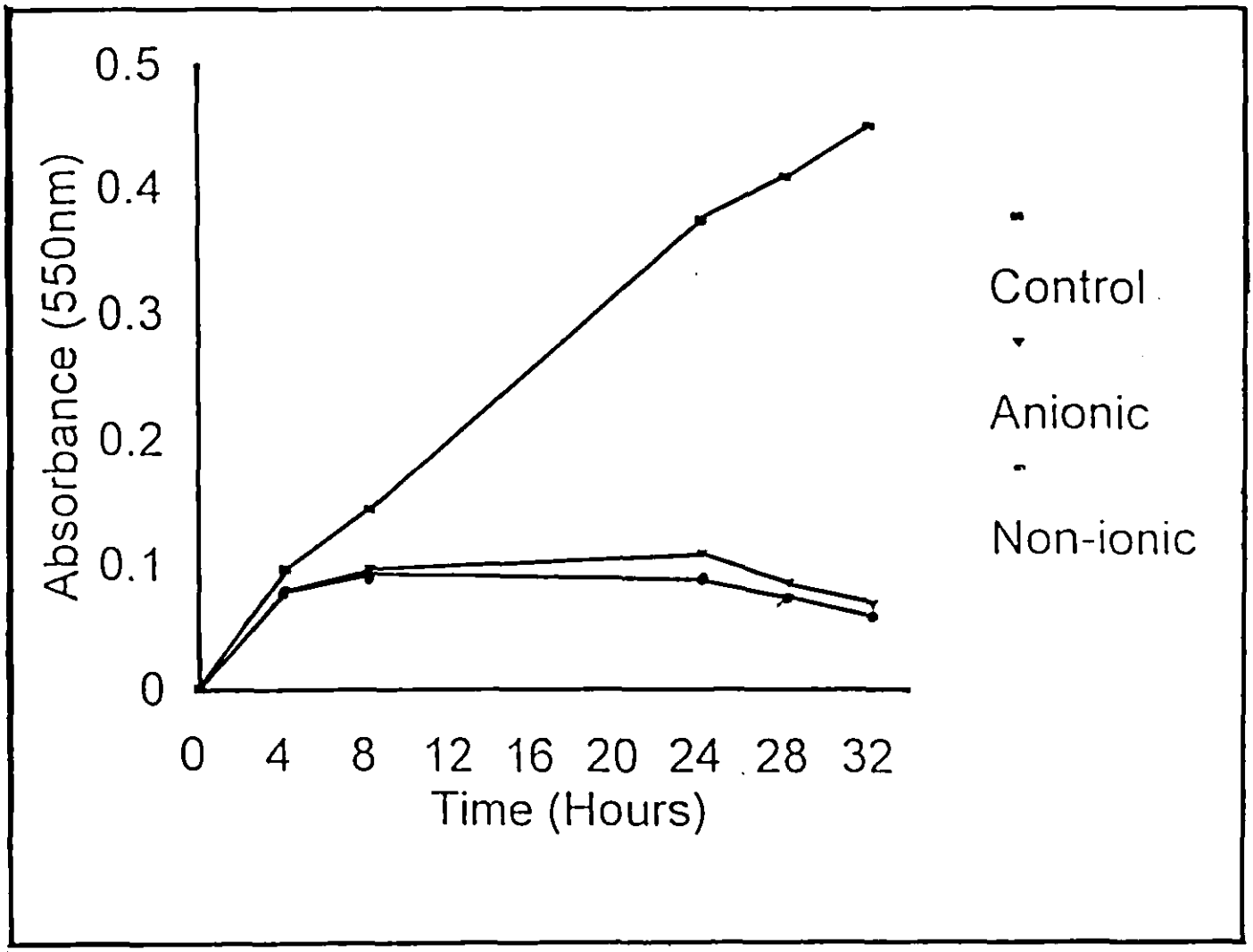

Figure 2 Absorbance measurements of *Ps aeruginosa* adherence to a glass tube over a period of 32h in the presence of 20 ppm of surfactant

Prevention of Ps. aeruginosa adhesion to 3CR12 metal coupons and a glass tube by the use of surfactants:

All the surfactants tested, inhibited *Ps. aeruginosa* adhesion significantly, when compared to the control (Fig. 1 and Table 1).

TABLE 1. *Pseudomonas aeruginosa* adhesion to the 3CR12 coupons, after treatment with the anionic and nonionic surfactants for 32h at a concentration of 20 ppm. The DAPI-staining technique was used for monitoring adhesion. 40

TREATMENT	NUMBER OF ATTACHED CELLS/cm^2	STANDARD DEVIATION	INHIBITION OF ATTACHMENT (% OF CONTROL)
Control	1.24 X 10^5	333	
Anionic surfactants:			
Product C1 (Chemserve)	7.88 x 10^3	370	93.64%
Product C35 (Chemserve)	7.86 x 10^3	290	93.66%
Product B3 (Buckman)	7.89 x 10^3	260	93.63%
Nonionic surfactants:			
Product B6 (Buckman)	6.54 x 10^3	400	94.73%
Product C33 (Chemserve)	6.56 x 10^3	610	94.71%
Product C17 (Chemserve)	6.56 x 10^3	660	94.71%
Product C24 (Chemserve)	6.58 x 10^3	330	94.69%
Product A1 (Anikem)	6.53 x 10^3	450	94.73%

The number of attached bacteria increased during the first 8h of exposure in the presence of the nonionic (product B6) and the anionic (product C1) surfactant (Fig. 1). During the next 16h of exposure to the surfactants, the rate of bacterial adhesion slowed down and declined after 24h. After 32h, the number of attached bacteria remained below 1 X 10^4 bacteria / cm^2 for all the surfactants tested (Table 1) as long as the surfactant was continuously dosed, compared to the 1,24 x 10^5 bacteria /cm^2 of the control. Anionic and nonionic surfactant treatments, resulted in an average of 93% and 94% inhibition of bacterial attachment respectively (Table 1). When the dosing was stopped, the adhesion rate increased to resemble that of the control. For example: Product C1 resulted in a 93,64% inhibition of bacterial attachment and product B6 in a 94,73% inhibition of bacterial attachment compared to the control system, after a treatment period of 32h (Fig. 3 and 4). There was no significant difference amongst the effectivity of the different anionic surfactants as well as amongst the effectivity of the nonionic surfactants. The nonionic surfactants were significantly more effective in inhibiting bacterial attachment than the anionic surfactants. Even so, this difference is of no practical consequence (93% - 94%).

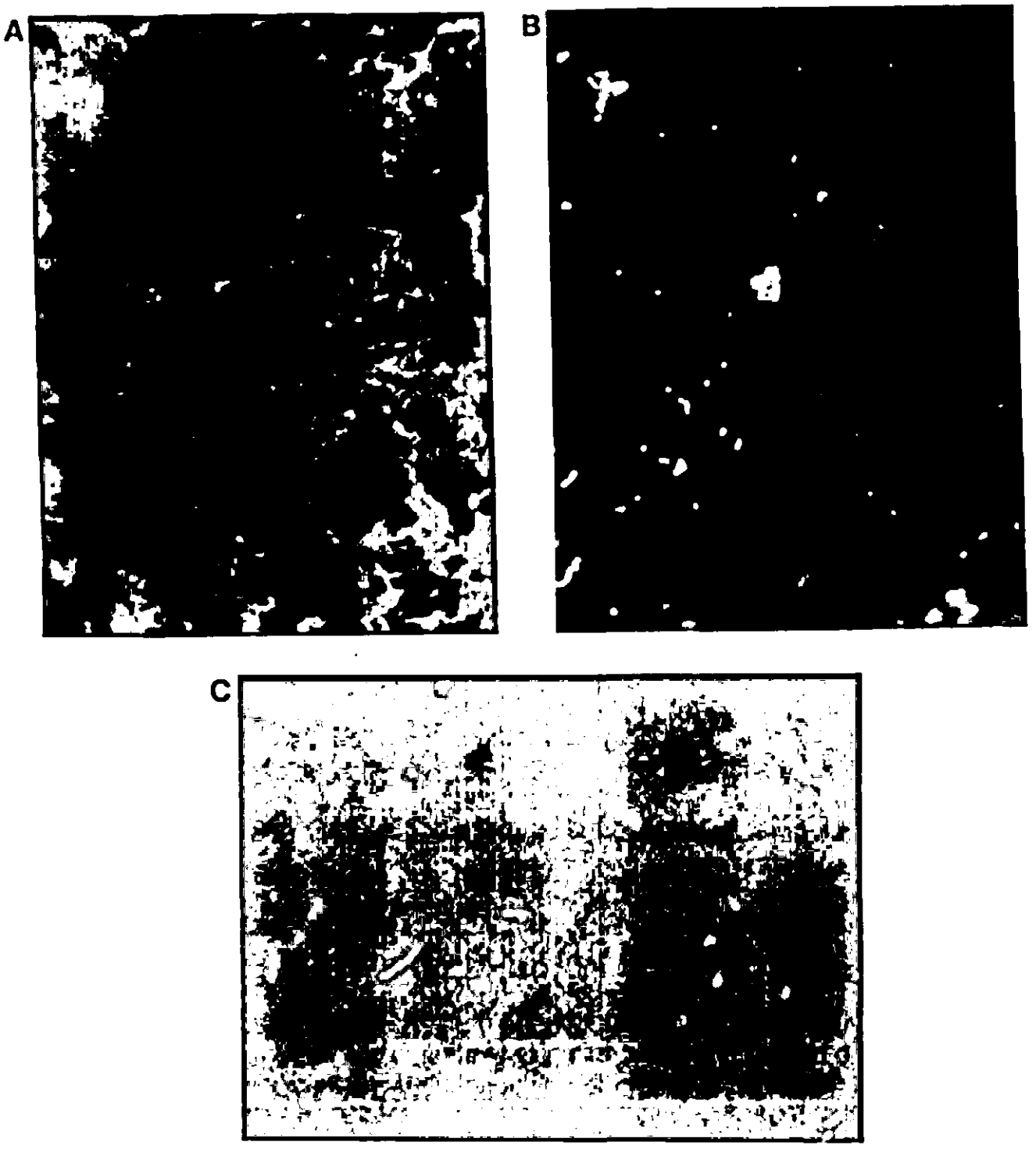

Figure 3. Number of DAPI-stained *Pseudomonas aeruginosa* cells adhering to the 3CR12 coupons over a period of 32 h, in (A) the absence of surfactants, (B) the presence of the nonionic surfactant (Product B6) and (C) in the presence of the anionic surfactant (Product C1). (800 x Magnification)

The absorbance measured for bacterial biofilm formation after exposure of the system to the anionic (C1) and nonionic (B6) surfactant for 32h was 0,069 and 0,060 respectively. The absorbance for the control system after 32h was 0,445 (Fig. 2). The inhibition of bacterial attachment to the glass tube after treating the system for 32 h was therefore 86,5% and 84,4% respectively for the products B6 and C1.

The percentage inhibition of bacteria adhering to the coupons after exposing the system to product B6 and C1 over a time period of 32h using the DAPI-staining technique and spectrophotometric evaluations, was compared. There was no significant difference ($p = 0,437$) between the results obtained using these different methods.

Removal of adhering bacteria:
All the surfactants tested resulted in a significant decrease in bacteria attached to the coupons (Table 2). The anionic surfactant (product C35) resulted in a 63,46% decrease in attached cells compared to the control.

TABLE 2. Removal of pre-attached *Pseudomonas aeruginosa* to 3CR12 coupons, by exposing the biofilm to anionic and nonionic surfactants at a concentration of 20ppm for 32h. The DAPI-staining technique was used for monitoring adhesion.

TREATMENT	NUMBER OF ATTACHED DEVIATION CELLS/cm^2	STANDARD	% REMOVAL
Control	4.68×10^5	337	
Anionic surfactants:			
Product C1 (Chemserve)	1.68×10^5	333	64.10%
Product C35(Chemserve)	1.71×10^5	577	63.46%
Product B3 (Buckman)	1.70×10^5	468	63.67%
Nonionic surfactants:			
Product B6 (Buckman)	9.15×10^4	207	80.45%
Product C33 (Chemserve)	9.14×10^4	115	80.47%
Product C17 (Chemserve)	9.16×10^4	307	80.43%
Product C24 (Chemserve)	9.13×10^4	106	80.49%
Product A1 (Anikem)	9.18×10^4	248	80.38%

There was an 80,45% decrease in the number of attached bacteria when the nonionic surfactant (product B6) was used (Fig. 5). After the initial decrease in attached cells, the number of bacteria adhering to the surfaces remained constant at this lowered colonization rate, until dosing was stopped and the number of attached bacteria increased once again. The nonionic and anionic surfactants, resulted in an average of 80,44% and 63,74% decrease in attached bacteria, respectively. There was no significant difference between the effectivity of the different anionic surfactants as well as the effectivity of the nonionic surfactants. The nonionic surfactants were significantly more effective in removing pre-attached cells from the coupons than the anionic surfactants . These results were obtained after treatment of a 7 d mature biofilm of *Ps. aeruginosa* with the surfactants. The decrease in the attached bacterial cells after treating the mature biofilm with the nonionic (B6) and the anionic (C1) surfactants are shown in Fig. 6 and 7. A withdrawal of the bacteria into clumps was noticed with the DAPI-staining technique, leaving areas of the surface uncolonized (Fig. 6). If the bacteria were allowed to adhere to the coupons for only 2h, after which dosing of the surfactants was started, complete removal of the biofilm occurred (Fig. 6 and 7).

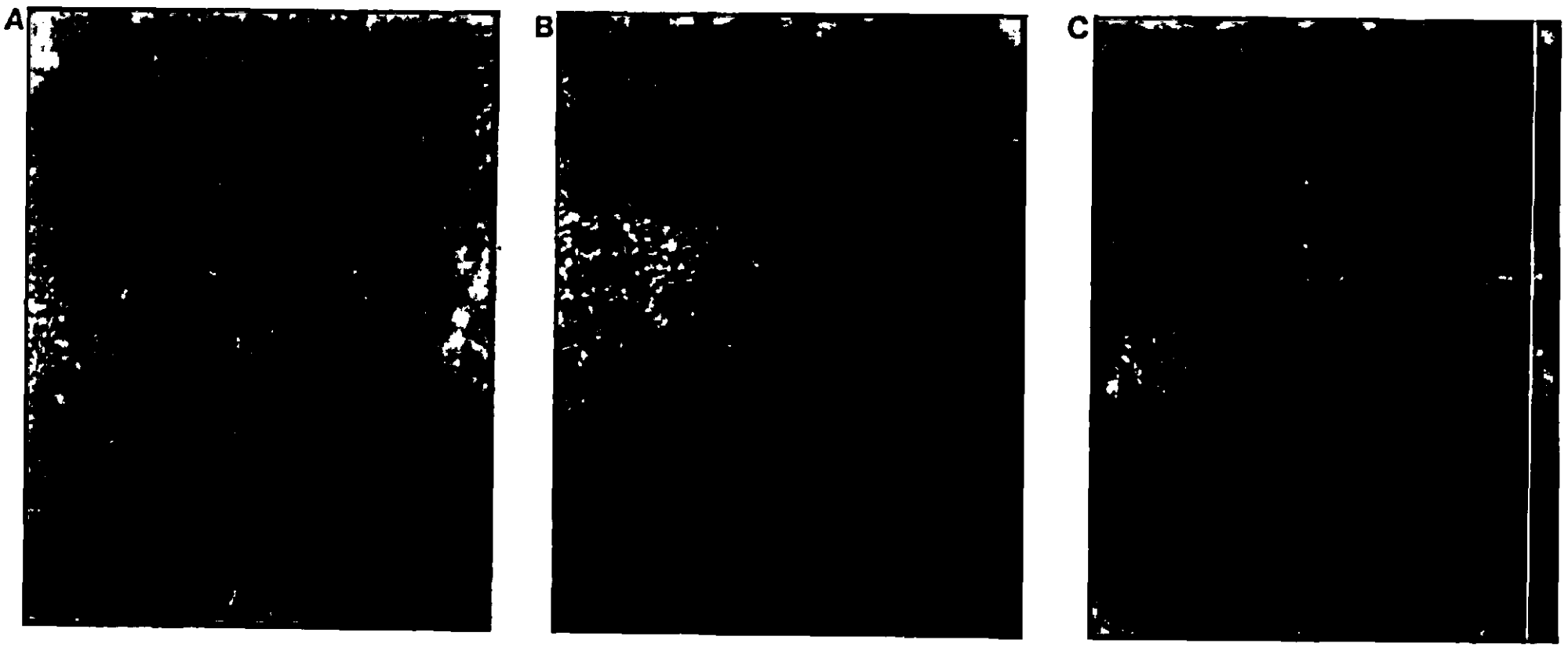

Figure 6. DAPI stains of a 168 hour *Pseudomonas aeruginosa* biofilm on 3CR12 coupons (A) before any addition of surfactants, (B) after treatment with the nonionic surfactant (Product B6) for another 24h and (C) after treatment with the anionic surfactant (Product C1) for 24h.

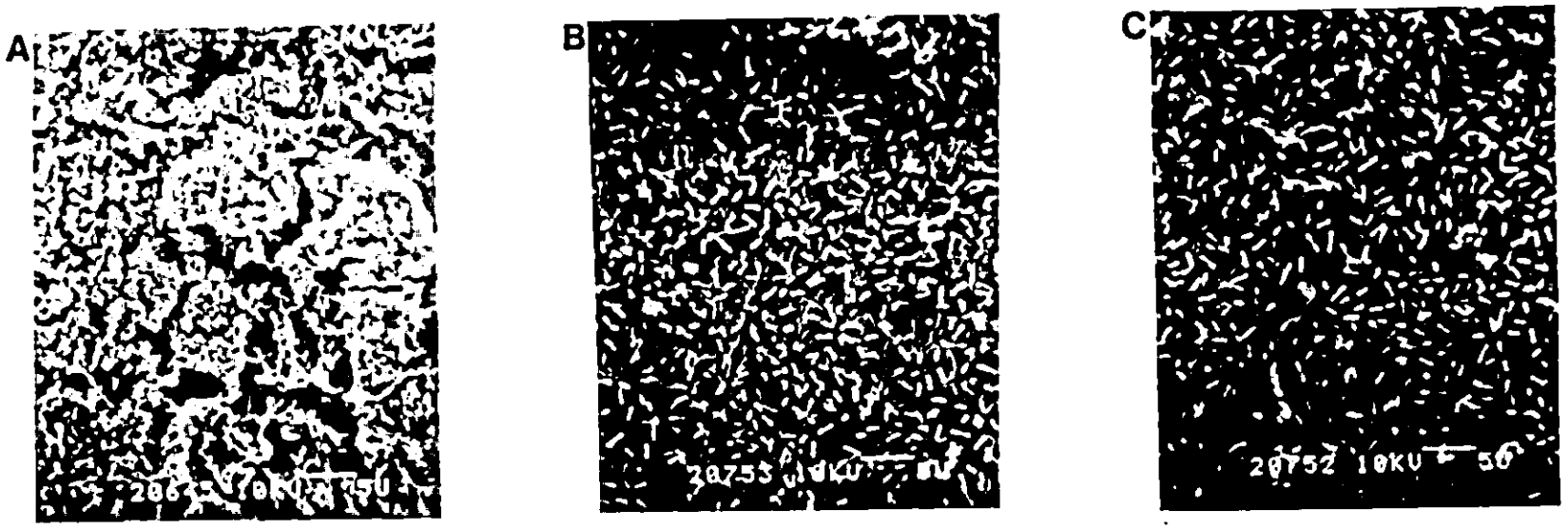

Figure 7. SEM photomicrographs of a 168h *Ps. aeruginosa* biofilm on a 3CR12 coupon (A) before treatment, (B) 24h after continuous exposure to a nonionic surfactant (Product B6) and (C) 24h after continuous exposure to an anionic surfactant (Product C1). (Bars = 5μm)

DISCUSSION

Prevention of bacterial adhesion:
All the nonionic and anionic surfactants had anti adhesive effects on bacteria attachment. There was no significant difference in the effectivity of the different anionic surfactants nor amongst the effectivity of the different nonionic surfactants. The nonionic surfactants were more efficient in preventing *Ps. aeruginosa* adherence to the surface, than the anionic surfactants. The nonionic surfactants all gave more than 94% inhibition of microbial adhesion, when compared to the untreated control (Table 1). The anionic surfactants all resulted in more than 93% less adhesion, when compared with the control (Table 1). This difference in effectivity is of no practical value for industrial water systems. These results compared well with the results of Whitekettle (1991) who found that the surfactants all gave more than 90% inhibition of microbial adhesion. According to Whitekettle (1991) nonionic surfactants had the greatest efficacy as inhibitors of microbial adhesion. Compounds capable of reducing surface tension by 20 mN/m, showed the greatest efficacy as inhibitors of microbial adhesion (Whitekettle, 1991).

Removal of a mature biofilm:
Removal of the mature biofilm proved a more difficult task. Both the surfactants removed pre-attached bacteria, but total removal of the biofilm did not occur. A significant decrease in the number of adhered bacteria was observed within the first 6h of treatment. After this initial decrease, the number of attached bacteria stabilized and remained constant. This decrease in attachment could be the result of the removal of loosely attached bacteria. The remaining bacteria were irreversibly attached and could not be removed by these compounds at the given concentration. According to Marshall *et al.* (1971) bacterial adhesion occurs in two phases: Reversible sorption where bacteria are held weakly near the surface and are readily removed by washing. Irreversible sorption which involves the firm adhesion of the bacteria to the surface and which can not be removed by washing. Special cell surface structures (eg. fibrils or polymers) form strong links between cell and solid surface (Van Loosdrecht *et al.*, 1990). These polymers are essential for the development of surface films (Geesey, 1982). Bacteria that are allowed to adhere strongly and form adhesive polymers (exopolysaccharides) are more difficult to remove (Marshall *et al.*, 1971). A higher concentration of surfactants might therefore be necessary to remove these communities. By allowing the bacteria to adhere to the surfaces for only 2h, complete removal of the adhered bacteria occurred. Similar results were reported by Paul and Jeffrey (1985).

Removal of bacteria adhering to the coupons for 168h were significantly more difficult to remove than bacteria that were allowed to adhere to the coupons for only 2h. As soon as the dosing of the surfactants was stopped, the number of attached bacteria increased. The effectivity amongst the different anionic surfactants to remove pre-attached bacteria did not differ significantly. Neither was the effectivity amongst the nonionic surfactants significantly different in removing pre-attached bacteria. The nonionic surfactants, however, were significantly more effective in removing pre-attached bacteria, than the anionic surfactants. The nonionic surfactants were more efficient for removing pre-attached *Ps. aeruginosa* cells (80,38% - 80,49%) than anionic surfactants (63,67 - 64,10%).

Spectrophotometric results for the evaluation of dispersants for prevention of bacterial adhesion to solid surfaces compared well with the corresponding DAPI - staining technique results (p = 0,437). Absorbance proved to be a quick and easy method to evaluate the surfactants for their ability to prevent bacterial adhesion to surfaces. It is an inexpensive and uncomplicated alternative to techniques requiring labourious counting of microorganisms, for evaluating surface-active compounds. Using absorbance to determine the efficacy of surfactants proved to be a reliable method for evaluating surfactants for their prevention of biofouling.

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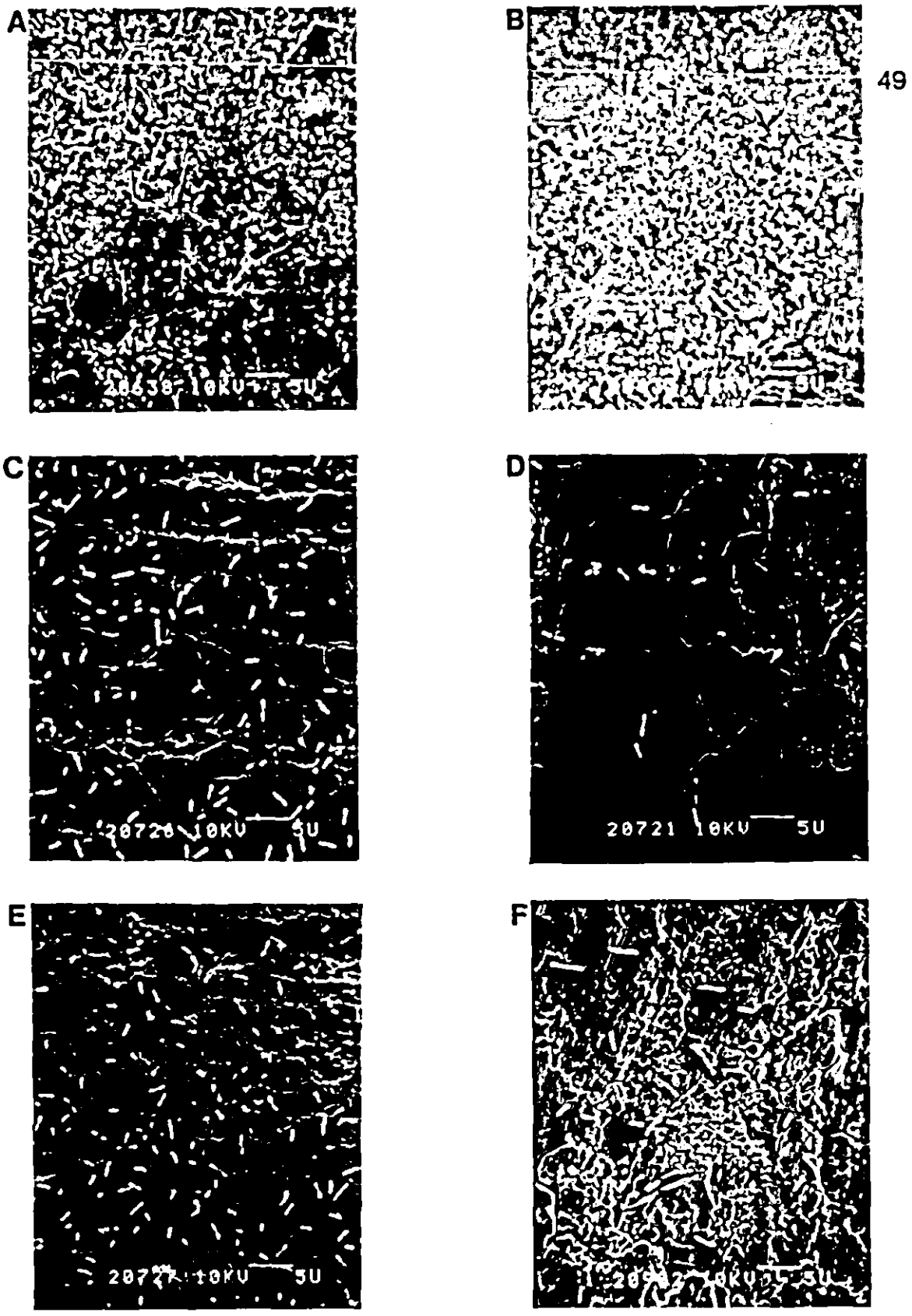

Figure 4. SEM photomicrographs of *Ps. aeruginosa* attaching to 3CR12 metal coupons after exposure for (A) 24h and (B) 32h in the absence of the surfactants, (C) 24h and (D) 32h in the presence of the nonionic surfactant (Product B6) as well as (E) 24h and (F) 32h in the presence of the anionic surfactant (Product C1). Concentrations of 20 ppm were used. (The bars = 50m)

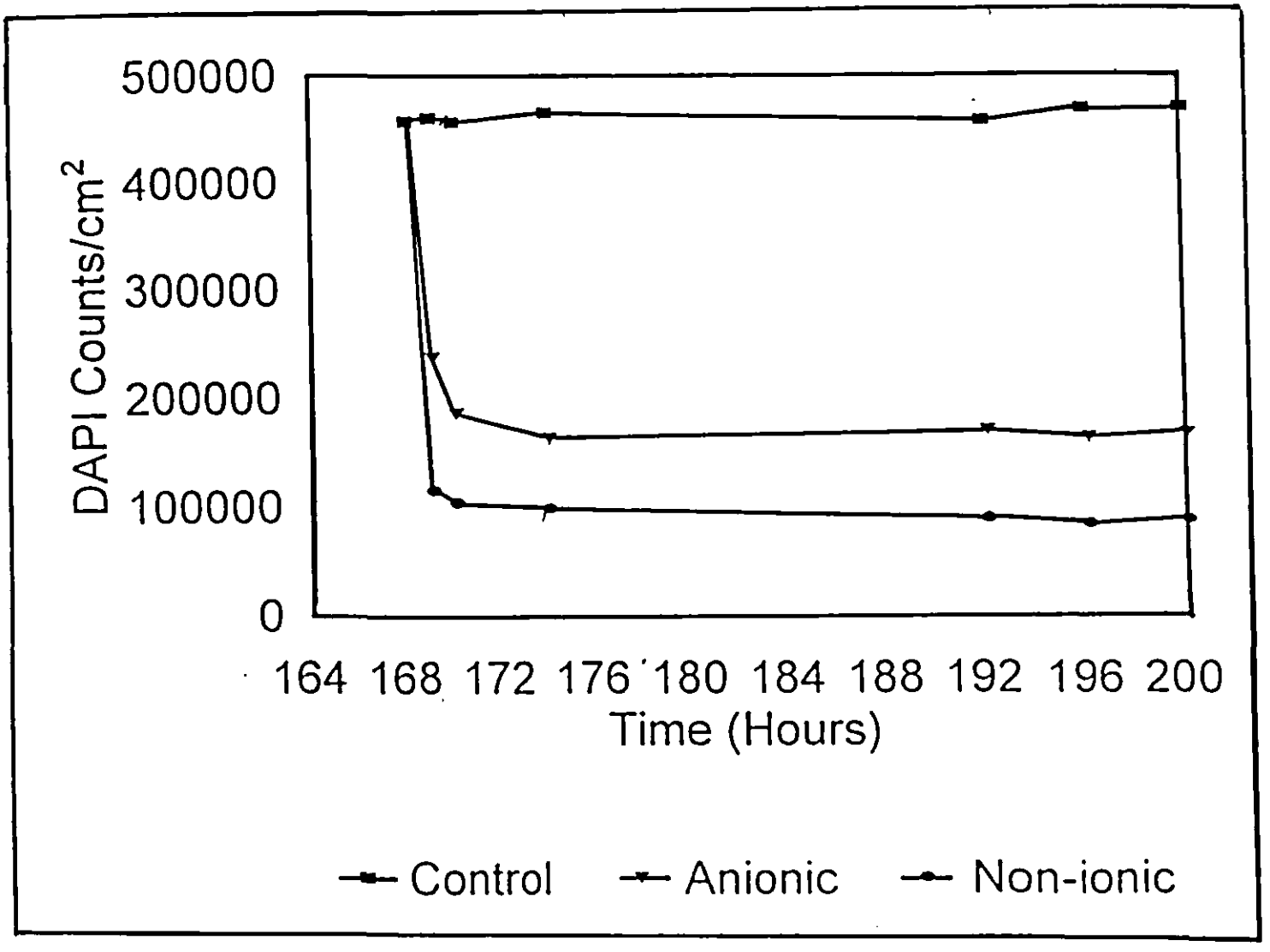

Figure 5. The number of DAPI-stained *Ps. aeruginosa* cells adhering to the 3CR12 coupons over a time period of 32h after treatment with the surfactants at 20 ppm. Treatment started after cells were allowed to attach for 168 h.

CHAPTER 5
THE SIMULTANEOUS USE OF A NONIONIC SURFACTANT AND AN ISOTHIAZOLONE BIOCIDE FOR CONTROLLING *Pseudomonas aeruginosa* ADHESION TO 3CR12 COUPONS

INTRODUCTION

Bacteria can adhere to almost any surface in any submerged aquatic environment in which they are present (Allison and Sutherland, 1987). Biofilms capture inorganic and organic molecules from the bulk liquid, making it available as nutrients for the organisms in the biofilm (Costerton and Lappin-Scott, 1989). Marshall *et al.* (1971) indicated that an initial reversible phase of sorption of bacteria to solid surfaces is followed by a time-dependant, and possible growth dependant, irreversible phase of sorption of some of the bacteria. The irreversible phase is associated with the production of extracellular fibrils that anchor the cell to solid surfaces (Marshall and Cruickshank, 1973). The extracellular polymers consist of a varying ratio of protein to polysaccharide mass of between 0 and 10 (Cloete *et al.*, 1989). These extracellular polymers form a tangled mass of fibres termed a biofilm (Cloete *et al.*, 1989). Biofilms form particularly rapidly in flowing systems where a regular supply of nutrients is provided to the bacteria Marshall, 1992). Biofilm formation in water cooling systems results in increasing biomass deposition (Whitekettle, 1991) causing fluid flow resistance, loss of heat exchange and microbially induced corrosion (Marshall, 1992).

Since no plastic or metal proved to be resistant to bacterial colonization, control of biofouling is a study field receiving much attention. Biofilm formation can be controlled by reducing organic nutrients in bulk water (Costerton and Lappin-Scott, 1989). This is however not possible in industrial water cooling systems. Water shortages forces the industries to recycle and reutilise water (Bondonno *et al.*, 1989). Recirculation of water concentrates dissolved and suspended substances, decreasing the water quality (Cloete et al., 1992). Unwanted biofilms are therefore controlled, with mixed success, using biocides or by incorporating toxic heavy metals into antifouling paints (Marshall, 1992). Bactericides attack targets of cell function, placing the bacterium under stress (Brözel and Cloete, 1992). Bacteria within the biofilm, therefore, develop increased resistance to biocides on repeated dosing. Repeated biocide applications favour growth of bacteria producing copious amounts of extracellular polysaccharides (EPS) that protects the cell from the biocide (Sakagami *et al.*, 1989). This results in higher concentrations of these toxic materials needed to inhibit the growth of attached organisms (Characklis and Dydek, 1976). Increasing biocide concentrations are costly, result in increasing environmental burdens and threaten non-target organisms (Whitekettle, 1991).

In recent years, the emphasis has shifted to the use of surfactants. These compounds are chemically synthesized and their surfactant properties result from the presence of both hydrophobic and hydrophilic regions on the same molecule (Fiechter, 1992). Surfactants are important constituents of biocides and are employed to achieve both uniform wetting of the surface to be treated and have an additional cleaning effect (Cloete *et al.*, 1992). Paul and Jeffrey (1985) proposed that the anti adhesive effect of Triton X-100 on bacteria was due to adsorption of the dispersant on the bacterial and substratum surfaces, masking hydrophobic groups on the two surfaces, and thereby preventing hydrophobic interactions. Humphries *et al.* (1986) proposed an alternative mechanism for the anti adhesive effect of surfactants. The specific modes of adsorption and orientation of nonionic surfactants on hydrophobic as opposed to hydrophilic substrata result in the inhibition of bacterial attachment to the substrata. Adsorption of the surfactant on the bacterial surface plays no significant role (Whitekettle, 1991). Irrespective of the debate on the anti-adhesive mechanisms of surfactants, they do have excellent anti adhesive effects. Surfactants resulted in more than 93% inhibition of *Pseudomonas aeruginosa* adhesion to solid surfaces, compared to the untreated control (Chapter 4).

Laboratory and field studies carried out by Lutey *et al.* (1989) showed that a biodispersant was effective in removing established biofilms and resulted in an increase in the number of planktonic bacteria. Dispersion of the bacteria into the bulk water prior to the addition of a biocide, would render the use of biocides more effective. It is unlikely that surfactants will have any mutagenic effects on bacteria, or that microorganisms would become resistant to the action of a surfactant. Surfactants are also less costly than biocides. The use of surfactants has become common practice, especially in the water cooling systems. They are used together with biocides to achieve optimum removal of the biofilm in these systems. The use of surfactants in conjunction with biocides to control biofouling and microbially induced corrosion (MIC) in industrial cooling water systems was more effective than the use of biocides alone (Poulton, 1993). The objective of this study was therefore to evaluate the simultaneous use of a nonionic surfactant and the biocide, isothiazolone, on biofilm formation, using a laboratory evaluation system.

MATERIALS AND METHODS

Culture used:
A *Pseudomonas aeruginosa* strain, isolated by Brözel and Cloete (1992) from cooling water systems in a previous study, was used.

Chemicals used:
A nonionic surfactant (Product B6) and an isothiazolone biocide were used. Both were obtained from South African suppliers.

Evaluation of the combined use of the surfactant and biocide in preventing Ps. aeruginosa adhesion to 3CR12 coupons:
The compounds were evaluated for their ability to prevent *Ps. aeruginosa* from adhering to 3CR12 coupons over a period of 32 h. The influence of both the surfactant and the biocide on

bacterial attachment were evaluated separately as well as in combination. The continuous flow-through system (Chapter 3), connected to the Pederson device (Pedersen, 1982), was used for these evaluations. *Ps. aeruginosa* was cultured in R2A broth (Reasoner and Geldreigh, 1985) for 24 h ar room temperature. The reservoir with 4 *l* R2A broth was inoculated with 20 ml of the *Ps. aeruginosa* culture and allowed to grow for another 24 h, before circulation of the culture through the Pedersen device with sterile coupons was initiated. The planktonic growth of the bacteria was monitored throughout the course of the experiment, and maintained at 1,0 - 2,8 x 10^8 cfu/ml by the continuous feed of sterile medium into the system. The surfactant was fed continuously into the system together with the sterile medium at a final concentration of 20 ppm. The biocide wasadded to the reservoir at the beginning of the test as well as continuously through the sterile medium at a final concentration of 25 ppm. Controls, where the coupons were exposed to the bacteria and media, without the addition of the biocide or surfactant, were included in the experiments. Biocide application to the system treated with both biocide and surfactant was discontinued. This experiment was also repeated by discontinuation of the surfactant.

The continuous flow-through system and modified Pedersen device used for the experiments are described in chapter 3. Biofilm formation was monitored using scanning electron microscopy (SEM) and the 4,6 - diamidino - 2 - phenylodole (DAPI) staining technique.

Evaluation of the use of the surfactant and biocide in combination for the removal of a mature Pseudomonas aeruginosa biofilm:
The ability of the chemicals to remove attached bacteria, was tested according to the method of Paul and Jeffrey (1985). *Ps. aeruginosa* was grown in R2A broth at room temperature to a density of 1,0 - 2,5 X 10^8 cells/ml. Sterile 3CR12 coupons were exposed for 24 h to 20 ml of this *Ps. aeruginosa* culture. Prior to exposure to the bacteria the coupons were prepared by the method of Wolfaardt *et al.* (1991). The resulting biofilm could not be removed by vigorous washing with water. Exposure of the coupons to the bacteria for 2 h, resulted in complete removal of attached cells by vigorous washing with water as was described by Paul and Jeffrey (1985). This demonstrated that an exposure time of 24 h resulted in the formation of a mature biofilm. After the short exposure period of 2 h, bacteria did not adhere irreversible and produced no exopolysaccharides to form a mature biofilm. The metal coupons, covered with a 24 hour mature *Ps. aeruginosa* biofilm, were exposed for 8 h to the surfactant and biocide at concentrations of 20 and 25 ppm, respectively. The surfactant and biocide were added separately and simultaneously to the petri dishes to evaluate their combined ability to enhance removal of the mature biofilm. Controls, where the coupons with the 24 hour *Ps. aeruginosa* biofilm were exposed to the sterile medium without the addition of neither the biocide nor surfactant, were included in the experiments.

RESULTS

Prevention of bacterial adhesion:
There was an initial increase in bacteria attachment to the coupons during the first 4 h of exposure to the bacteria and the surfactant and biocide. The attachment of bacteria in the

control system continued to increase for up to 32 h. Bacterial attachment rates started to decrease after 4 h in the systems treated with the biocide, surfactant and biocide surfactant combination (Fig. 1). In the system treated with either the surfactant or biocide, the number of adhered bacteria remained constant for 16h and decreased only after 24 h to 28 h (Fig. 1). However, when the surfactant and biocide were used in combination, there was an immediate decrease in the number of attached *Ps. aeruginosa* after 4 h. The number of bacteria adhering continued to decrease during the next 16 h, and then remained constant. After exposure for 32 h, 5,5 x 10^3 cells / cm^2 adhered to the coupons in the system exposed to both the surfactant and biocide simultaneously, compared to 6,5 x 10^3 cells /cm^2 and 7,3 x 10^3 cells / cm^2 respectively when the surfactant and biocide were dosed separately. In the control system 1,24 x 10^5 cells /cm^2 adhered after 32 h (Fig. 1,2 and 3). Using both the surfactant and the biocide in combination, a 95,5% decrease in adhesion, compared to a 94,1% and a 93,4% decrease for the surfactant and the biocide respectively, occurred.

The number of attached bacteria, stayed constant with discontinuation of biocide dosing in the system where both the biocide and surfactant were used simultaneously. However, if the dosing of only the surfactant was discontinued, the number of adhering bacteria, increased slightly by 0,9 -1,2 x 10^3 cells / cm^2, and then remained constant (Fig. 4)

Removal of a mature biofilm:
An immediate decrease in the number of attached bacteria occurred during the first 2h after the nonionic surfactant and biocide were introduced simultaneously compared to the control. A decrease in the number of bacteria adhering, occurred only after 8 h when the biocide and surfactants were used separately. A reduction of 93% and 91% occurred after 8 h, respectively when the surfactant and biocide were applied separately. After 8 h a reduction of 97,7% occurred in the number of adhering *Ps. aeruginosa* when using a combination of surfactant and biocide. When the biocide and nonionic surfactant were used separately, an 88% and 91% reduction in bacteria adhering occurred after 24 h respectively. Enhanced decrease in the number of adhered bacteria to the metal surfaces when the 24 h biofilm (Fig. 6a) were treated with the surfactant and biocide in combination (Fig. 6d. and Fig. 7) compared to separate treatments with the surfactant and biocide. A decrease in number of bacteria adhering, after the 24 h biofilm (fig. 7a) was treated with both the biocide and surfactant for 8 h (fig. 7b), occurred.

DISCUSSION

Prevention of bacterial attachment:
The nonionic surfactant and the isothiazolone biocide both controlled biofouling of the 3CR12 coupons by *Ps. aeruginosa* when used separately, resulting in a 94,1 and 93,4% reduction respectively. The number of adhered bacteria decreased by 95,5% when the surfactant and biocide were used in combination. In the systems treated separately with the biocide and surfactant, *Ps. aeruginosa* attachment increased during the first 4 h of exposure and then remained constant for up to 24 h before the number of attached cells decreased (Fig. 1). However, when both these compounds were used simultaneously, the number of adhered bacteria decreased rapidly within 4 h and remained low during the 32 h treatment period. The initial increase in the number of attached bacteria was the result of an initial low concentration

of the compounds in the system during the first 4 h of the experiment. The amount of surfactant and biocide increased during the next 4 h to the desired concentrations, as the compounds started to circulate through the system. When the surfactant and biocide were not dosed continuously, the number of adhered bacteria increased rapidly, from 5,5 x 10^3 cells / cm^2 to 1,65 x 10^4 cells / cm^2 within 4 h. The number of attached bacteria stayed constant with discontinuation of the biocide application in the system where both the biocide and surfactant were dosed simultaneously (5,5 x 10^3 cells /cm^2). When dosing of the surfactant was discontinued, the number of adhered bacteria, increased slightly (7,8 x 10^3 cells / cm^2) and then remained constant. Surfactant application is therefore important to keep adhering bacteria numbers low. Planktonic growth in the reservoir remained undisturbed during the 32 h period, through continuous supply of cells through the system, from the reservoir. The reduction in biofilm formation was therefore not the result of a decrease in planktonic population and demonstrated the direct effect of the biocide and surfactant on the attached *Ps. aeruginosa* cells.

Removal of a mature biofilm:
Both surfactant and biocide were able to remove a mature 24 h *Ps. aeruginosa* biofilm. When the surfactant and the biocide were used in combination, the number of attached bacteria decreased more rapidly (within the first 2 h), resulting in a much faster removal of the biofilm compared to the separate use of the biocide and surfactant. The biofilm could not be removed entirely, with either of the methods used. The biocide and surfactant resulted in a 93,4% and 94,1% decrease, respectively, in bacterial attachment, compared to the control. The simultaneous use of the surfactant and biocide resulted in a 97% decrease in the number of bacteria adhering to the coupons, compared to that of the control. This decrease occurred over a period of 4 h, whereas the decrease in the attached bacteria occurred over a period of 6 h when the products were used separately. A biofilm control program including the use of both a surfactant and a biocide would result in more rapid removal of attached bacteria than using the surfactant and biocide seperately. The colonization of the microorganisms could therefore be brought under control in less time, resulting in a more efficient control of biofouling. The best results for inhibition of *Ps. aeruginosa* attachment to solid surfaces as well as removal of a *Ps. aeruginosa* biofilm were obtained by the simultaneous application of a nonionic surfactant and a isothiazolone biocide. The different effects of the different control programmes on biofouling were demonstrated using a laboratory continuous flow through system.

Since the results presented here, were based on tests performed under laboratory conditions with pure cultures, direct application to full scale industrial systems is not possible. Conditions and microbial combinations in industrial systems vary. Current evaluation of biocides, surfactants and simultaneous use of different combinations of a biocide and surfactant in industrial systems is impractical, time consuming and expensive. Incorrect control programs can lead to the development of resistant microorganisms and aggravate the problem. This laboratory method, is therefore an easy, fast and safe way of screening compounds and for evaluation of different combinations for further large scale industrial experimentation.

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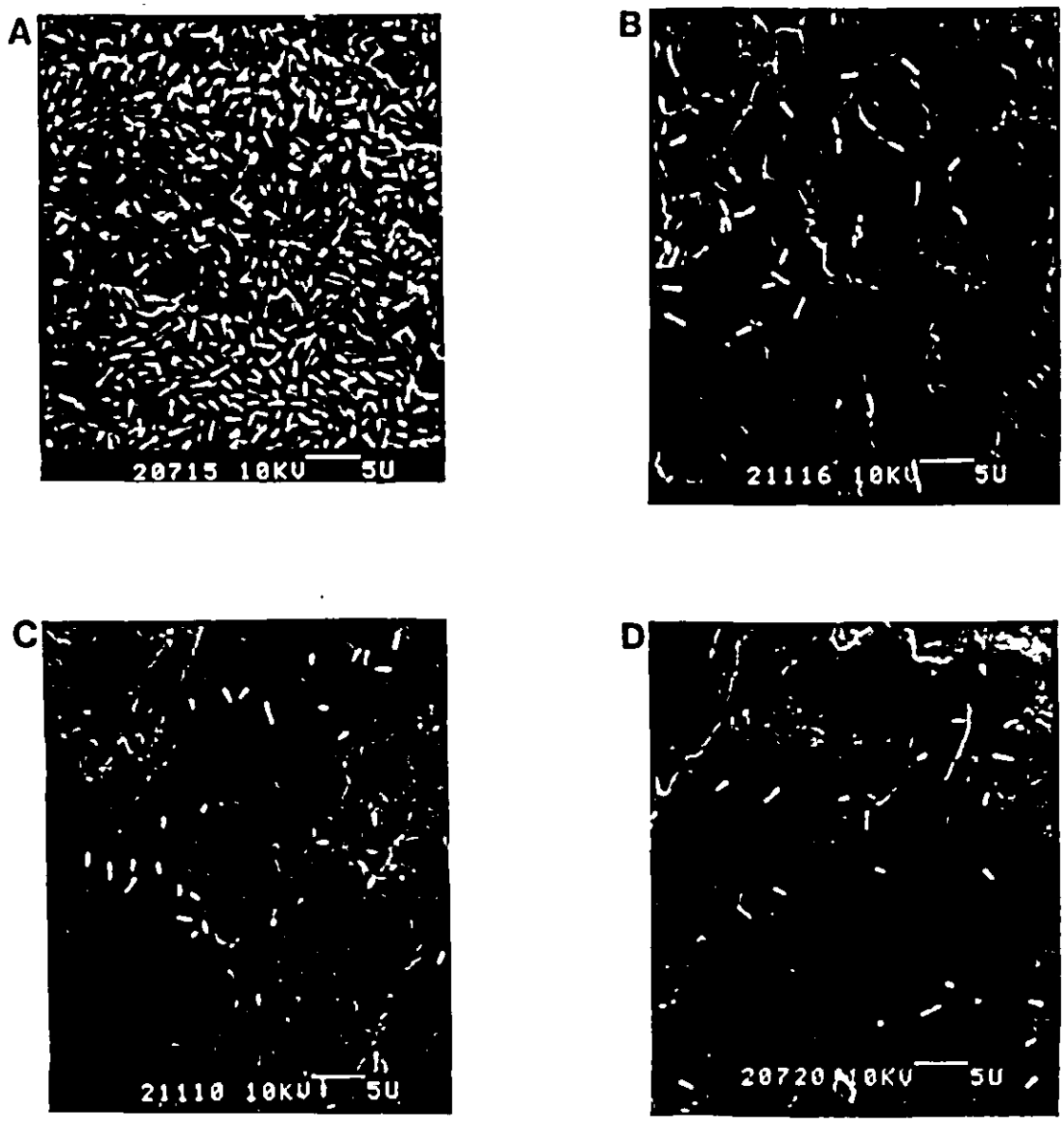

Figure 3. SEM photomicrographs of adhering *Ps. aeruginosa* cells after 32 h. The untreated control is represented in (A), the biocide treated coupon in (B), the surfactant treated (C) and the surfactant and biocide treated coupon in (D). (The bar = 5□m)

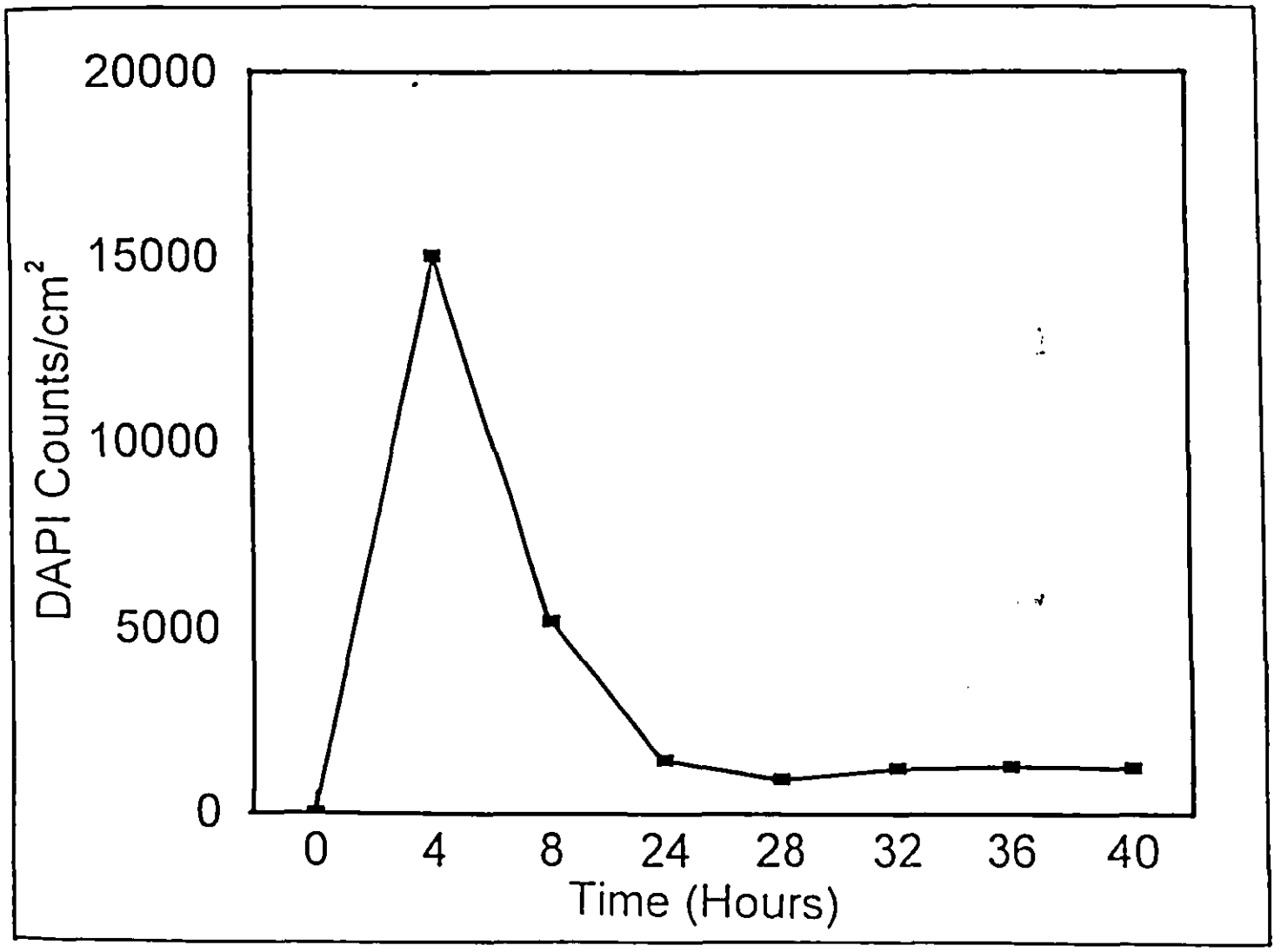

Figure 4. DAPI-stained *Ps. aeruginosa* cells adhering, after discontinuation of the surfactant treatment program. At 28 h of exposure of the bacteria to both the biocide and surfactant, the addition of the surfactant stopped, and the number of adhering bacteria increased slightly over the next 4 h , and then remained constant.

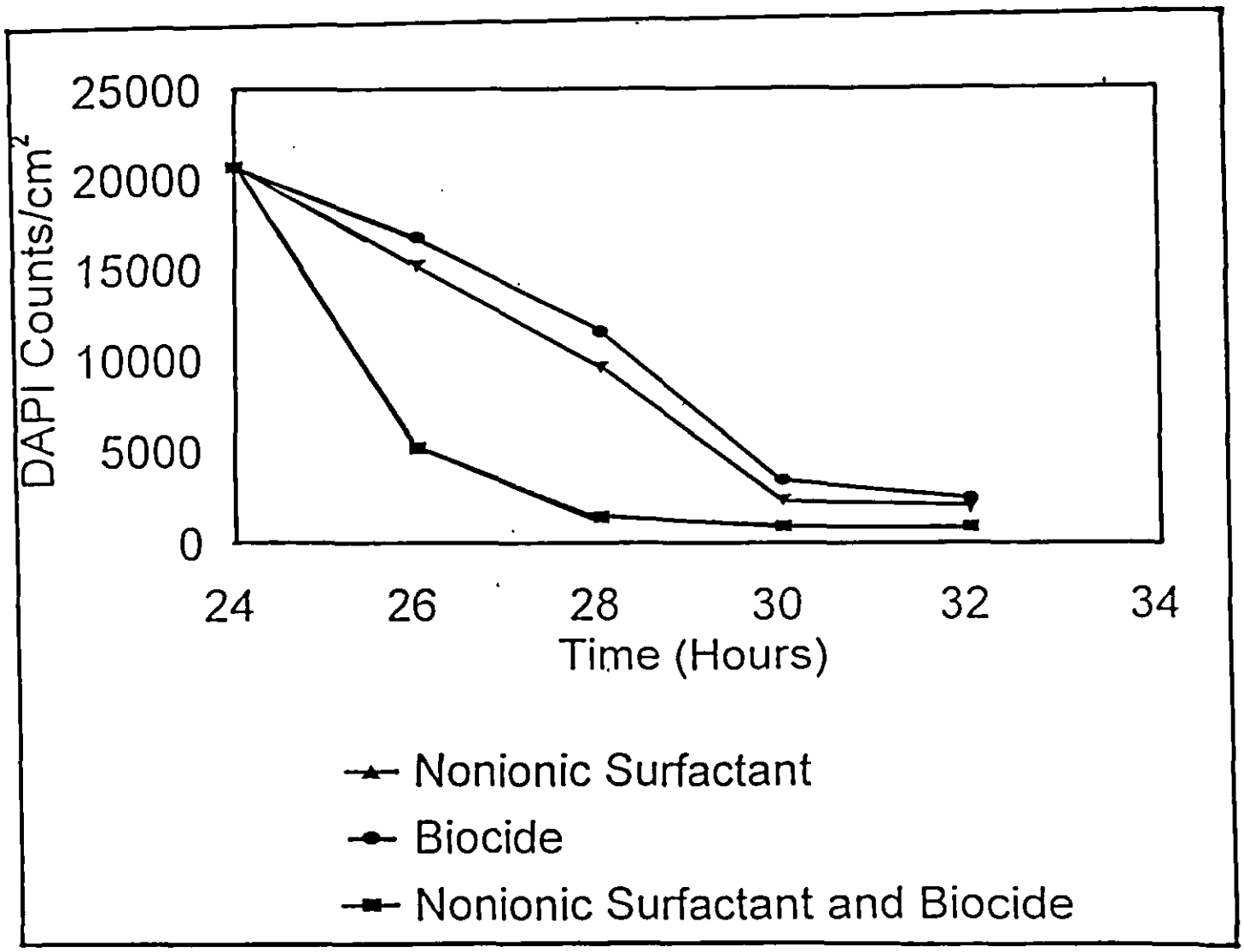

Figure 5. The removal of a DAPI-stained *Ps. aeruginosa* biofilm with the nonionic surfactant and isothiazolone biocide separately, and in combination. The biofilm was formed over a period of 24 h, before treatment commenced. The control (not shown) continued to increase over the time period indicated, to an number of more than $1,2 \times 10^5$ cells/cm^2 at 32 h.

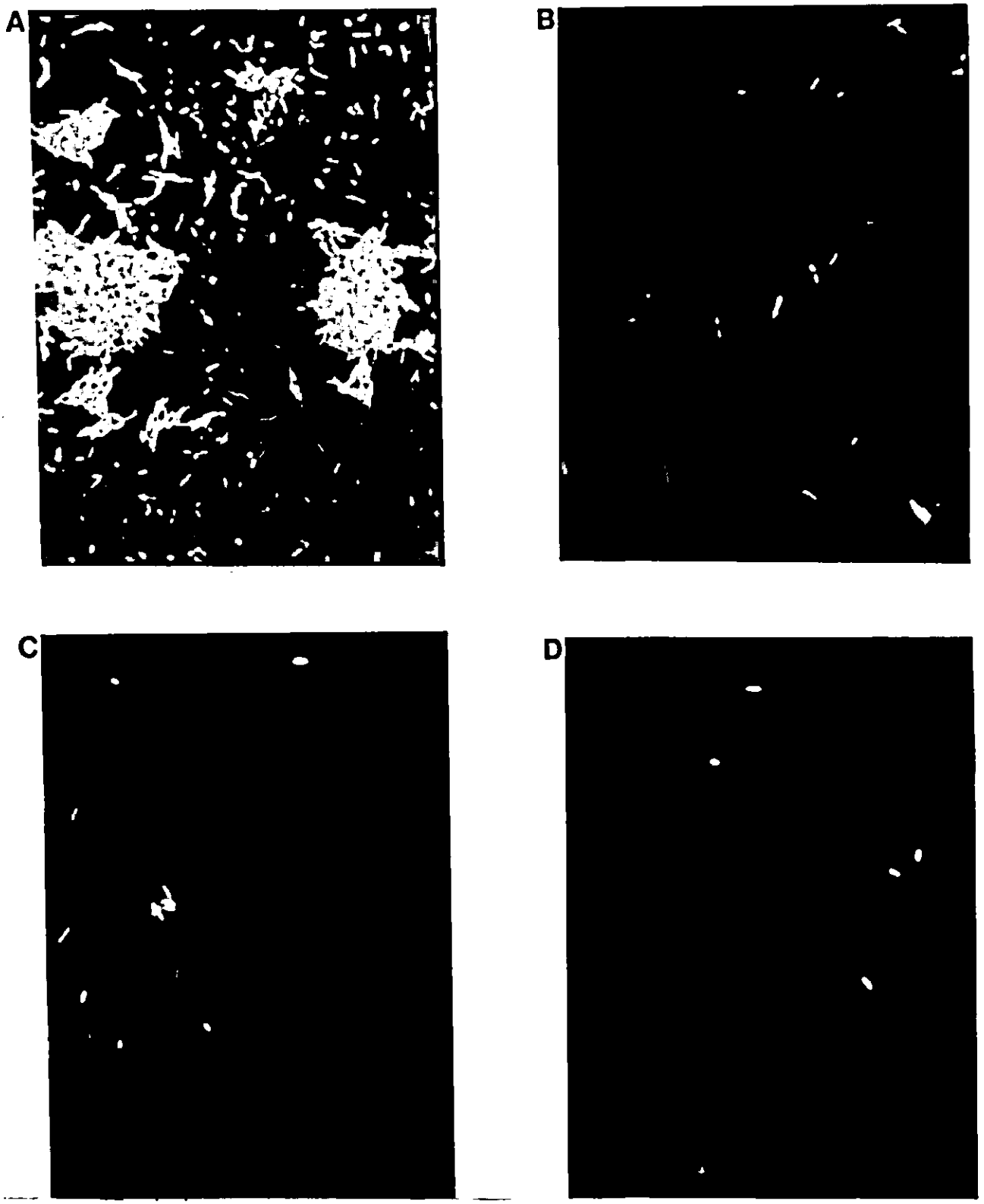

Figure 6. DAPI-stained *Ps. aeruginosa* cells adhering for 24 h, before the treatment of the surfactant and biocide started (A) and after treatment of the 24 hour mature *Ps. aeruginosa* biofilm with the biocide (B), the surfactant (C) as well as with both the nonionic surfactant and Isothiozolin biocide (D) for 24 h at concentrations of 20 and 25 ppm respectively. (800 x magnification)

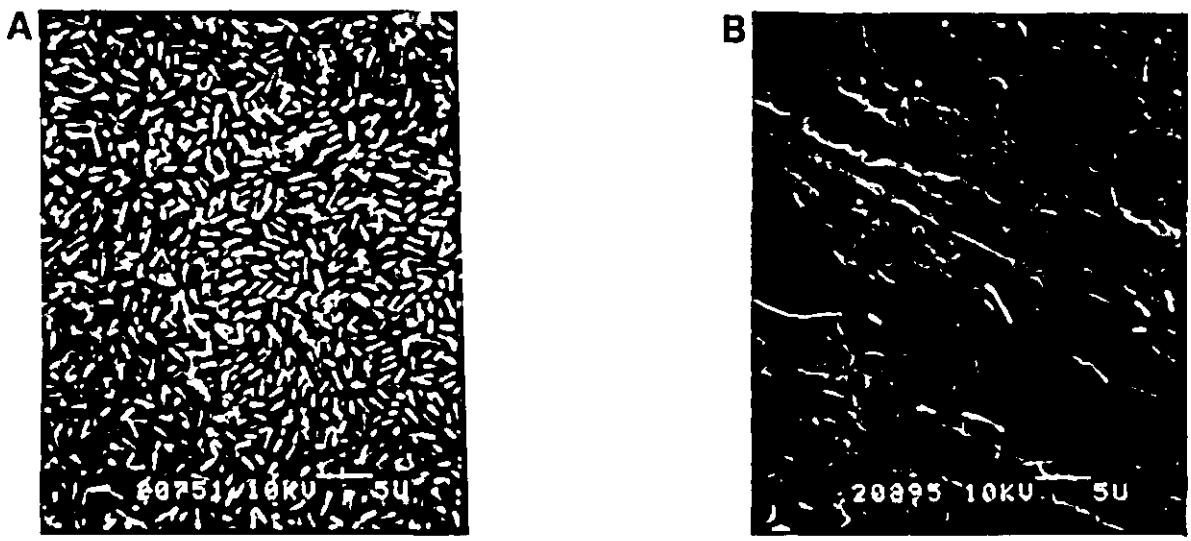

Figure 7. SEM photomicrographs of the 24 hour mature *Ps. aeruginosa* biofilm on the 3CR12 coupons (A). More than 95% of the mature biofilm was removed, using the surfactant and biocide in conjunction (B). The concentration of the surfactant and biocide is 20 and 25 ppm respectively. (Bar = 5□m)

CHAPTER 6
ENZYMATIC CONTROL OF *Pseudomonas aeruginosa* ADHESION TO 3CR12 COUPONS

INTRODUCTION

Heukelakiam and Heller (1940) first suggested that microorganisms attach to surfaces in order to survive. Adsorbed nutrients are concentrated and thus more accessible to the bacteria after attachment to the surfaces (Zobell, 1943). This adhesion and subsequent metabolism lead to the formation of biofilms (Brözel and Cloete, 1992). Biofilm formation results in increasing biomass deposition (Whitekettle, 1991), fluid flow resistance, loss of heat exchange and microbially induced corrosion (MIC) (Marshall, 1992).

There are currently five approaches for controlling biofouling: (I) bacteria are chemically killed by the use of biocides; (ii) bacteria are physically removed by a process known as pigging ; (iii) biofilms in heat exchangers can be removed by the ice nucleation process; (iv) biofilms are dispersed by dispersants; and (v) biofilms are degraded by enzymes (Cloete *et al.*, 1992).

Methods such as pigging and ice nucleation are time consuming, expensive and need to be done on a regular basis to be effective. Biocides only react with the outer portion of the biofilm, leaving a healthy and substantial bacterial community on the surface that rapidly regrows (Marshall, 1992). Bacteria within the biofilm develop increased resistance to the biocide on repeated dosing (Cloete *et al.*, 1992). Higher concentrations of toxic materials are needed to inhibit the growth of sessile organisms, which is costly, results in increased environmental burdens and threatens non-target organisms (Whitekettle, 1991).

Dispersants were shown to restrict the formation of biofilms to clean surfaces (Whitekettle, 1991), and are less costly than biocides. It is unlikely that microorganisms would become resistant to the action of surfactants. Surfactants are important constituents of biocides and are employed to achieve both uniform wetting of the surface to be treated and have an additional cleaning effect (Cloete *et al.*,1992). Using biodispersants in conjunction with biocides to control biofouling and MIC in cooling water systems was shown to be more effective than the use of biocides alone (Poulton, 1993). Chemical treatment is very costly and dosing needs to be done on a regular basis. More recent developments in biofouling control involves enzymes. There is however little published information available on the possible use of enzymes for the control of biofilm formation or bacterial attachment. The object of this paper was therefore to determine the efficacy of an enzyme to remove or prevent *Pseudomonas aeruginosa* adhesion to 3CR12 coupons, using a laboratory system.

MATERIALS AND METHODS

Organism used:
Pseudomonas aeruginosa, isolated from an industrial cooling water system (Brözel and Cloete, 1992), was cultured in R2A broth, for 24 h at room temperature. These bacterial cultures were used in all subsequent experiments.

Enzyme used:
The enzyme, CM 2XL, obtained from a South African supplier (Novo Nordisk), was used in this study.

The Continuous flow-through system and modified Pedersen device used for the experiments are described in chapter 3. Biofilm formation was monitored using scanning electron microscopy (SEM) and the 4,6 - diamino - 2 - phenylidole (DAPI) staining technique.

Evaluation of the degrading enzymes for their ability to prevent Pseudomonas aeruginosa from adhering to the 3CR12 coupons:
The enzyme was tested for its ability to prevent *Ps. aeruginosa* adhering to 3CR12 coupons over a period of 32 h. The enzyme was fed continuously into the continuous flow through system together with the sterile medium at a final concentration of 0,5%. Controls, where the coupons were exposed to the bacteria and media, without the addition of enzyme, were included in the experiment.

Evaluation of the enzyme for the removal of a mature Pseudomonas aeruginosa biofilm:
A mature *Ps. aeruginosa* biofilm was formed using the continuous flow through system connected to the Pedersen device with the 20 sterile 3CR12 coupons. The coupons were exposed to the media and bacteria alone for a period of 48 h, which resulted in a mature biofilm that could not be removed with vigorous washing with water. The enzyme was then added to the sterile medium and fed into the continuous flow through system for another 32 h.

RESULTS

Prevention of bacterial adhesion:
The number of sessile bacteria adhering to the solid surfaces of the control coupons increased over 32 h to 1,24 x 10^5cells/cm^2 (Table 1). When the enzyme was dosed continually over the 32 h period the number of *Ps. aeruginosa* adhering to 3CR12 coupons decreased to 1,6 x 10^4 cells/cm^2. The number of planktonic bacteria present in the reservoir was monitored throughout the experiments and remained constant at 1,5 - 2,8 x 10^8 cfu/ml. There was an initial increase in the number of adhered bacteria during the first 4 h. After 8 h, the number of bacteria adhering to the coupons decreased to 5,8 x 10^3 cells/cm^2 compared to 2,39 x 10^4 cells/cm^2 in the control system (Table 1). Enzymatic treatment for 8 h resulted in a decrease of 95% in the number of bacteria adhering. A 98,8% decrease in the number of bacteria adhering, was obtained after an exposure time of 24 h to the enzyme, compared to the control system (Fig. 1). After exposure to the enzyme for 32 h ,there was a 99% decrease in the

number of bacteria adhering compared to the control (Fig. 2a).

TABLE 1. Enzymatic prevention of *Pseudomonas aeruginosa* adhesion to the 3CR12 coupons.

TIME (h)	NUMBER OF ATTACHED CELLS/cm^2 (untreated)	ATTACHED CELLS/cm^2 (treated with 0,5% enzyme)
0	0	0
4	1,3 X 10^4	8,2 X 10^3
8	2,4 X 10^4	5,8 X 10^3
24	8,1 X 10^4	1,5 X 10^3
28	1,1 X 10^5	1,3 X 10^3
32	1,24 X 10^5	1,2 X 10^3

Removal of a 48 h bioflim:
Figure 3b shows the number of bacteria removed from the mature biofilm (Fig. 3a) after 24 h of treatment with the enzyme. The number of bacteria adhering to the coupons, decreased by 89% after 24 h of exposure to the enzyme (Table 2). The SEM photomicrographs also demonstrate the removal of the biofilm, after 24 h of exposure to the enzyme (Fig. 4b) compared to the control (Fig. 4a).

TABLE 2. Enzymatic removal of pre-attached *Ps. aeruginosa* to the 3CR12 coupons.

TIME (h)	NUMBER OF ATTACHED CELLS/cm^2 (untreated)	ATTACHED CELLS/cm^2 (treated with 0,5% enzymes)
0	1,4 X 10^5	1,4 X 10^5
4	1,7 X 10^5	8,6 X 10^4
8	1,3 X 10^5	4,2 X 10^4
24	1,5 X 10^5	1,6 X 10^4

CONCLUSION

The effect of the enzyme on the adhesion of *Ps. aeruginosa* to the 3CR12 coupons was studied using the continuous flow-through system. Adhesion of the bacteria, decreased by 99,9% compared to the untreated system, when treatment with the enzyme started before biofilm formation.

The enzyme did not result in the complete removal of a mature biofilm. Only 89% of the pre-attached bacteria were removed by exposure of a mature biofilm to the enzyme. This could be the result of the presence of the exopolysaccharide, forming a protective layer around the adhering bacteria. The degrading enzyme was more effective in preventing bacteria adhering to clean surfaces, than removing mature biofilms from surfaces. Enzymes are, however, still expensive and therefore not cost effective to find industrial application.

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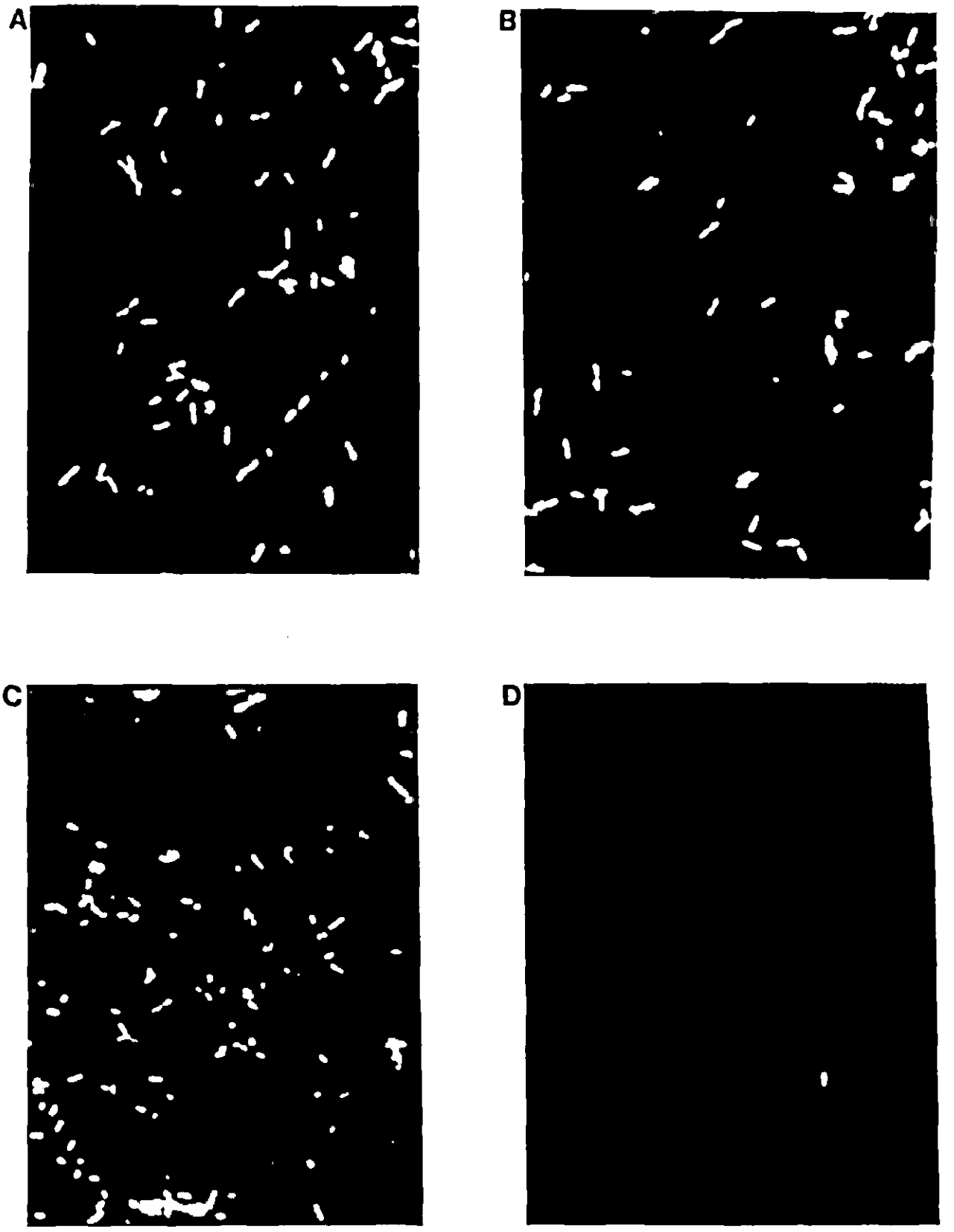

Figure 1. DAPI-stained *Ps. aeruginosa* adhering at (A) 4 h, (C) 8 h, (E) 24 h and (G) 32 h. The decreased number of adhering cells after exposure to the enzyme, at the corresponding times are shown in B, D, F and G respectively. (800 x magnification)

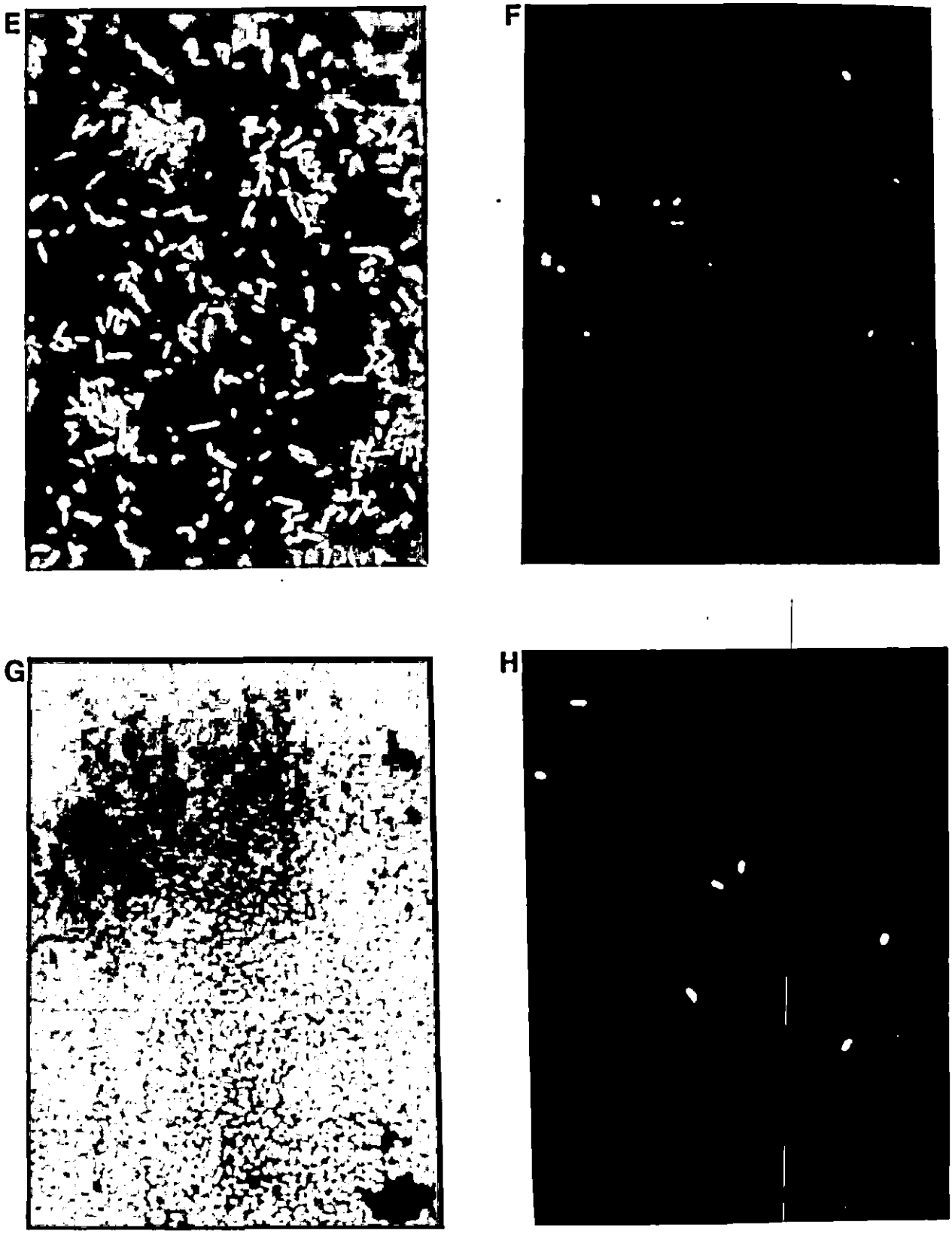

Figure 1. Continued

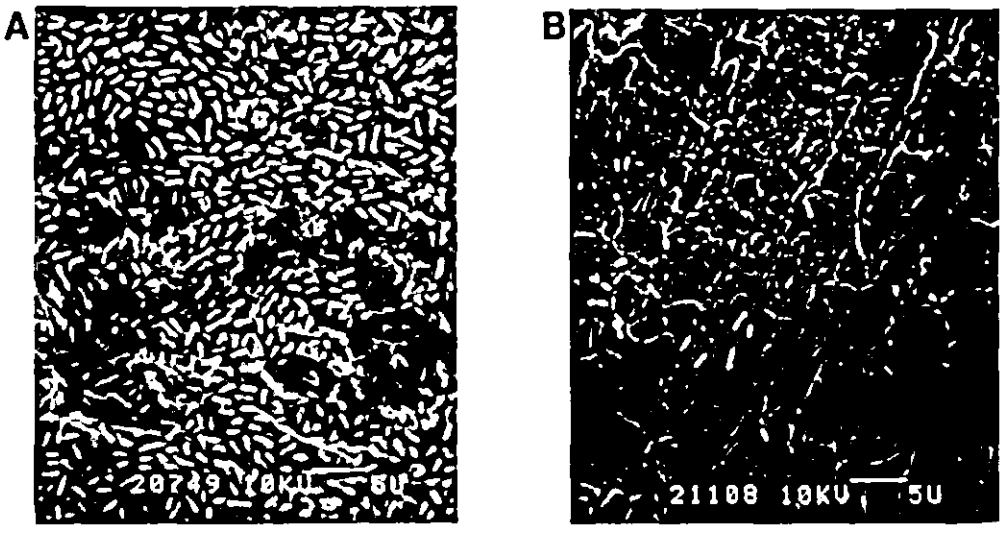

Figure 2. The SEM photomicrographs of *Ps. aeruginosa* attachment at 32 h, in the control (A) and (B) the system treated with the enzyme for 24 h. (Bar = 5□m)

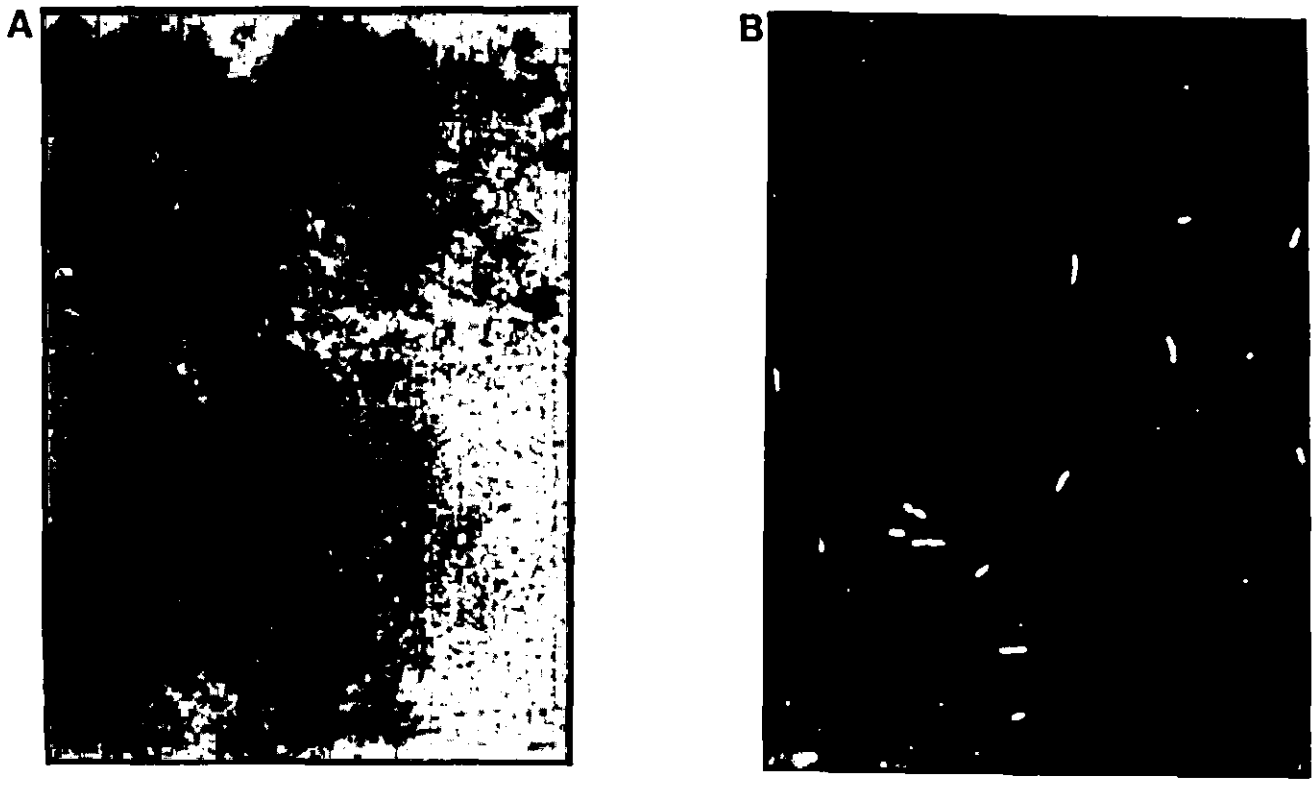

Figure 3. *Ps. aeruginosa* DAPI-stained mature biofilm (A) and (B) the biofilm after treatment with the enzyme for 24 h. Magnification at 800 times.

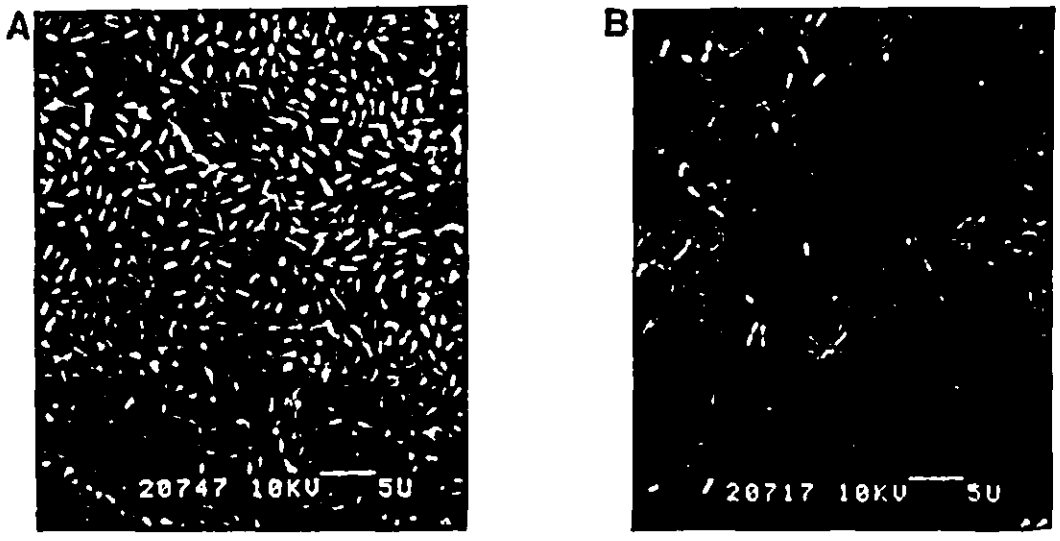

Figure 4. SEM photomicrographs of a *Ps. aeruginosa* mature biofilm (A) and (B) the biofilm after treatment with the enzyme for 24 h. (Bar = 5□m)

CHAPTER 7

CONCLUSIONS

Various techniques are available to study biofouling and the effect of biocides, surfactants and degrading enzymes on the adhesion of microorganisms to solid surfaces. Major drawbacks of currently used techniques to study biofouling are that bacteria must be counted, whether on culture plates or under the microscope. A continuous circulating batch culture system was therefore designed to study biofouling spectrophotometrically. The use of absorbance to determine biofouling, eliminates the need for culturing and counting of bacteria. The direct measurement of absorbance correlated well with the total counts obtained using the DAPI-staining technique (r^2 = 0,925). This technique proved a reliable alternative to techniques requiring labourious counting of microorganisms.

Industries control biofouling using biocides. Biocides only react with the outer portion of the biofilm, leaving a healthy and substantial bacterial community on the surface that rapidly regrows. Bacteria within the biofilm also develop increasing resistance to biocides on repeated dosing. More recently, surfactants were employed to control bacterial adhesion to solid surfaces. Surfactants result in both uniform wetting of the surface and have an additional cleaning effect. It is unlikely that surfactants will have any mutagenic effects on bacteria, or that microorganisms would become resistant to the action of surfactants.

The efficacy of a range of nonionic and anionic surfactants, in preventing and removing *Pseudomonas aeruginosa* adhesion to solid surfaces were evaluated. The prevention of attachment of *Ps. aeruginosa* cells to a glass surface, using surfactants, was monitored spectrophotometrically. There was no significant difference ($p = 0.437$) in the percentages of inhibition of bacterial attachment to solid surfaces using surfactants, when comparing the DAPI - staining technique with spectrophotometric evaluations. All the surfactants resulted in more than 90% inhibition of adhesion of *Ps. aeruginosa* to the surfaces. There was no significant difference amongst the efficacy of the different anionic surfactants ($p \square 0.18$) as well as amongst the efficacy of the nonionic surfactants ($p \square 0.16$). The nonionic surfactants were significantly more effective ($p \square 0.001$) in inhibiting and removing bacterial attachment than the anionic surfactants. However, the difference was small and of no practical significance to the industry. The nonionic surfactants were the most efficient in removing pre-attached bacterial cells. A decrease of more than 80% in the number of attached bacteria was obtained, compared to an average of 63% decrease using anionic surfactants. The amount of attached bacteria still remained high (1×10^5 bacteria / cm^2) after treatment of a mature biofilm. Using absorbance to determine the efficacy of surfactants to prevent bacterial adhesion therefore proved to be a reliable and rapid method.

When a biocide (isothiazolin) and nonionic surfactant were used in combination, the number of bacteria adhering to the solid surfaces, decreased by 95,5%. A 94,1% and 93,4% decrease in the number of bacteria attached were obtained respectively, using the nonionic surfactant and biocide. The biocide and surfactant resulted in a 93,4% and 94,1% decrease in the number

of attached bacteria in a mature biofilm, respectively, compared to the control. The simultaneous use of the surfactant and biocide resulted in a 97% decrease in the amount of bacteria adhering to a mature biofilm. A biofilm control program including the use of both a surfactant and a biocide would result in more rapid removal of attached bacteria than using the surfactant and biocide separately.

The enzymes studied prevented bacterial attachment to solid surfaces by 99%. A reduction of 89% in the number of bacteria adhering to the coupons occurred when a mature biofilm was treated with the enzyme. Since most surfaces in industries have already adhering bacterial communities, the use of a combination of surfactants and biocides will result in the best removal of these communities. Degrading enzymes had good anti-adhesive properties, but could not remove a mature biofilm. They are therefore best employed on clean surfaces to prevent microorganisms from adhering to the surfaces. Enzymes have not yet found uses in controlling biofouling in water cooling systems, because tehy are not cost effective.