UNIVERSITY OF MINHO
DEPARTAMENT OF BIOLOGICAL ENGINEERING

Use of biocides and surfactants to control *Pseudomonas fluorescens* biofilms

role of the hydrodynamic conditions

Dissertation for PhD degree in Chemical and Biological Engineering

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"Eu vos direi o que é a sabedoria e qual a sua origem, e não vos esconderei os seus mistérios; mas investigá-la-ei até à sua origem mais remota, e porei às claras o seu conhecimento e não me afastarei da verdade."

(Sab 6, 22)

A Nossa Senhora, à minha mãe, à Lúcia, ao pai-avô.

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Simões M. Pereira M. O. Vieira M. J. (2003) Effect of hydrodynamic conditions on the efficacy of aldehyde-based biocides *P. fluorescens* biofilms. Proceedings of the Euro Summer School – Improving Human Potential (FP5) BIOIMEB-Biofilms in Industry, Medicine & Environmental Biotechnology – Galway, Irland.

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Abstract

Biofilms constitute a protected growth modality that allows the bacteria to survive in hostile environments. The most common practice to eliminate unwanted biofilms is the application of antimicrobial agents. However, current disinfection practices show often inefficacy in the control of biofilms.

The main goals of this work were the development of effective strategies, based on the application of chemical agents, in order to control biofilms formed by *Pseudomonas fluorescens,* and the evaluation of the impact of the hydrodynamic conditions under which biofilms were formed, in the phenotypic characteristics of the biofilms, as well as, in their susceptibility to antimicrobial agents.

The antimicrobial agents tested were two non-oxidizing aldehyde-based biocides (*ortho*-phthalaldehyde - OPA and glutaraldehyde - GTA), two oxidizing biocides (sodium hydroxide - NaOH and sodium hypochlorite - SHC) and two surfactants (cetyltrimethyl ammonium bromide - CTAB and sodium dodecyl sulfate - SDS). The antimicrobial agents were selected due to their emergent and practical application in several industrial areas. The bacterial biofilms were developed on stainless steel surfaces in a flow cell reactor and in a bioreactor with a rotating device, being implemented different hydrodynamic conditions (laminar and turbulent flow) for biofilm formation. The action of the antimicrobial agents was assessed, mainly, by the determination of the biofilm respiratory activity, as well as, by the quantification of the biofilm mass removed, being tested several concentrations, contact times and strategies of application. The bacteria grown in biofilms under different flow regimes were phenotypically characterized in terms of outer membrane proteins expression (OMP), metabolic activity, biochemical composition and structure, being their phenotypes compared with bacteria in planktonic state. The respirometric method based on the assessment of the bacterial respiratory activity, the basic tool in the evaluation of the efficiency of the antimicrobial agents and in the characterization of the metabolic state of the bacteria, was validated by comparison with two reference methods (assessment of viability by "Live/Dead" stains and culturability in a solid medium).

Within the scope of this thesis, and for further comparison, it was assessed the antimicrobial properties of the biocides OPA and GTA and of the surfactants CTAB and SDS in the control of planktonic cells. These experiments revealed that, in the range of concentrations tested, OPA was more efficient in the bacterial respiratory inactivation than GTA, causing total bacterial inactivation. Concerning the surfactants, both caused reduction of the bacterial activity, but only CTAB caused total bacterial inactivation. However, the chemical agents reacted differently with the bacteria, being CTAB the unique that promoted cellular disruption. The bacterial phenotype was affected with the application of the chemical agents, since the OMP expression and the cellular pellet colour changed after chemical treatment. The presence of bovine serum albumin (BSA) in the bacterial cultures (in order to simulate dirty conditions found in industrial systems) reduced significantly the antimicrobial action of the several chemical agents.

Concerning biofilm formation in the flow cells, it was found that the flow regime and the sessile mode of life itself caused significant modifications in the metabolism, morphology and constitutive biochemical composition of the *P. fluorescens* cells.

The application of biocides (OPA and GTA) and surfactants (CTAB and SDS) to the biofilms developed in the flow cells, under different hydrodynamic conditions, revealed that, independently of the concentration, exposure time and strategy of application, every chemical agent was more effective in the inactivation of laminar biofilms

than turbulent biofilms, being OPA the only chemical that caused total inactivation. The entire chemicals tested exhibited poor ability in the removal of biofilms from the surfaces, independently of the flow regime under which biofilms were formed. It was verified a post-antimicrobial agents application effect for both biofilms, since after chemical treatment, biofilms recovered with the time their metabolic activity and viability, an evidence of the capability of biofilms to regrow. The comparison of these results with the ones obtained with the planktonic cells emphasises the higher resistance of biofilm microorganisms to disinfection when compared with their freely suspended counterparts. The results also underscore the inadequacy of planktonic testing methods for evaluating antimicrobial agents to be used as a means to control biofilms. This fact contradicts the presuppositions of the European Standard – EN 1276 (1997), where the bactericidal activity of disinfectants to be used in food, industrial, domestic and institutional areas are tested using cells in planktonic state.

The biofilms grown in the bioreactor with the rotating device showed an accentuated inherent mechanical stability, *i.e.,* a strong cohesion face to sudden changes in the surrounding hydrodynamic conditions. These biofilms were treated with OPA, GTA, NaOH, SHC, CTAB and SDS with the purpose to ascertain if after chemical treatment the mechanical stability of the biofilms (the biofilm behaviour face to external mechanical stress) was changed. It was found that biofilms previously treated with CTAB, NaOH, SHC, OPA and SDS (for concentrations near the critical micellar concentration) the biofilm mechanical stability decreased. Concerning GTA and SDS (for low concentrations), it was found an increase in the biofilm mechanical stability. These results highlight that even the synergistic chemical and mechanical treatments did not induce total biofilm eradication.

Finally, with the purpose to ascertain if a strain can represent a specie, the biofilm formation ability, as single or mixed populations, of two *P. fluorescens* native strains from a dairy industry was assessed using the flow cell reactor and the bioreactor rotating system. Similar characteristics were found comparing the biofilms formed by the different strains, as single or mixed biofilms. The phenotypic characteristics of the native strains were comparable with the ones obtained with the type strain, even when native strains were used to form mixed biofilms.

The results collected in this work allowed to conclude the role of the flow regime under which biofilms are formed in their susceptibility to antimicrobial agents, as well as, in the success/fallibility of procedures for biofilm control, fundamentally when extreme conditions are tested. The biofilms recovered their metabolic activity and viability, after chemical treatment, even when they presented merely residual activity. The submission of biofilms, previously exposed to antimicrobial agents, to hydrodynamic conditions different from the ones biofilms were developed did not cause total biofilm eradication from the surfaces contributing, therefore, for biofilm recalcitrance.

Resumo

Os biofilmes constituem uma forma de crescimento que permite a sobrevivência de bactérias em condições ambientais adversas. Os procedimentos mais comuns de controlo de biofilmes indesejáveis englobam a aplicação de agentes antimicrobianos. Contudo, esses procedimentos de desinfecção demonstram, frequentemente, alguma ineficiência na inactivação e eliminação dos biofilmes.

Este trabalho teve como principais objectivos o desenvolvimento de estratégias eficientes de controlo de biofilmes formados por *Pseudomonas fluorescens* recorrendo a produtos químicos antimicrobianos, e a avaliação do impacto das condições hidrodinâmicas usadas durante a formação de biofilme, nas suas características fenotípicas, bem como na sua sensibilidade aos agentes antimicrobianos.

Os agentes antimicrobianos testados incluiram dois biocidas não-oxidantes da família dos aldeídos (OPA e GTA), dois biocidas oxidantes (NaOH e SHC) e dois tensioactivos (CTAB e SDS), todos representando aplicações práticas e emergentes em vários sectores industriais. Os biofilmes bacterianos foram desenvolvidos sobre superfícies de aço inoxidável em células de fluxo e num bioreactor com sistemas rotativos, utilizando-se diferentes condições hidrodinâmicas (fluxo turbulento e laminar). O desempenho dos agentes antimicrobianos foi avaliado pela determinação da actividade respiratória dos biofilmes, bem como pela quantificação da massa de biofilme removida, tendo-se testado várias concentrações, tempos de contacto e estratégias de aplicação. As bactérias desenvolvidas em biofilme sob diferentes regimes de fluxo foram caracterizadas fenotipicamente em termos da expressão de proteínas da parede celular (OMP), actividade metabólica, composição bioquímica, e estrutura, e foram comparadas com as bactérias em suspensão. O método respirométrico baseado na determinação da actividade respiratória das bactérias, ferramenta básica na determinação da eficiência dos agentes antimicrobianos e na caracterização do estado metabólico das bactérias, foi validado pela comparação com dois métodos de referência (determinação da viabilidade recorrendo a corantes "Live/Dead" e caracterização da cultivabilidade em meio sólido).

No âmbito da dissertação, e para comparação posterior, começou-se por avaliar a capacidade antimicrobiana dos biocidas GTA e OPA e dos tensioactivos CTAB e SDS no controlo de células em suspensão. Estes ensaios revelaram que, na gama de concentrações testadas, o OPA foi mais eficiente na inactivação respiratória das bactérias do que o GTA, provocando a inactivação total das células bacterianas. Em relação aos tensioactivos, apesar de ambos causarem redução da actividade respiratória, somente o CTAB provocou inactivação total das células. Contudo, os vários agentes químicos reagiram com as bactérias de forma diferente, tendo o CTAB sido o único que provocou ruptura celular. O fenótipo bacteriano foi afectado com a aplicação dos agentes químicos, pois a expressão das OMP e a cor do "pellet" celular sofreram alteração. A presença de BSA (proteína de soro bovino) nas culturas bacterianas em suspensão (como forma de simulação de condições sujas encontradas em ambientes industriais) causou reduções significativas na acção antimicrobiana de todos os agentes químicos testados.

Relativamente aos ensaios de formação de biofilmes nas células de fluxo, verificou-se que o regime de fluxo e o próprio modo de vida séssil causaram alterações importantes no metabolismo, morfologia e composição bioquímica constitutiva das bactérias *P. fluorescens*.

Os ensaios de aplicação dos biocidas (GTA e OPA) e dos tensioactivos (CTAB e SDS) aos biofilmes formados nas células de fluxo, em condições hidrodinâmicas diferentes, revelaram que, independentemente da concentração, tempo de exposição e estratégia de aplicação, todos os produtos foram mais eficientes na inactivação

de biofilmes formados em regime laminar do que em regime turbulento, sendo o OPA o único químico a causar inactivação total. Todos os agentes químicos testados revelaram ser pouco eficientes na remoção de biofilme das superfícies, independentemente do regime de fluxo em que foram desenvolvidos. Verificou-se um efeito pós-aplicação do tratamento químico para ambos os tipos de biofilme, pois os biofilmes recuperaram com o tempo a sua actividade metabólica e a sua viabilidade, prova da capacidade de recrescimento dos biofilmes. A comparação destes resultados com os obtidos com as bactérias em suspensão reforça o fenómeno da maior resistência à desinfecção de microrganismos em biofilmes quando comparados com os mesmos em suspensão. Estes resultados, também, questionam o uso de testes em suspensão para avaliação de agentes antimicrobianos, principalmente, quando estes se destinam ao controlo de biofilmes. Este facto refuta os pressupostos existentes na Norma Europeia – EN 1276 (1997), em que a actividade bactericida de potenciais desinfectantes, para uso nas áreas alimentar, industrial, doméstica e institucional, é investigada com células em estado planctónico.

Os biofilmes desenvolvidos no bioreactor de sistema rotativo apresentaram uma acentuada estabilidade mecânica intrínseca, isto é, uma forte coesão face a alterações súbitas das condições hidrodinâmicas do meio circundante. Estes biofilmes quando tratados com GTA, OPA, NaOH, SHC, CTAB e SDS com o objectivo de investigar se após o tratamento químico sofreram alterações da sua estabilidade mecânica (o seu comportamento face a condições externas adversas). Verificou-se que os biofilmes previamente tratados com CTAB, NAOH, SHC, OPA e SDS (para concentrações próximas da concentração miceliar crítica) registaram uma diminuição da sua estabilidade mecânica. Em relação ao GTA e SDS (para concentrações baixas), verificou-se que contribuíam para o aumento da estabilidade mecânica do biofilme. Porém, mesmo a sinergia entre tratamentos químico e mecânico não provocou a erradicação total do biofilme.

Com o objectivo de averiguar se o comportamento de uma estirpe poderá representar o da espécie, determinou-se a capacidade de formação de biofilme por duas estirpes de *P. fluorescens* nativas de uma indústria de lacticínios, usando o reactor de célula de fluxo e o bioreactor de sistema rotativo. Da comparação das características dos biofilmes formados pelas duas estirpes individual e em cultura mista, concluiu-se que estes eram semelhantes. As características fenotípicas das estirpes nativas foram comparáveis às obtidas com a estirpe tipo, independentemente do modo de formação de biofilme (simples e misto).

Os resultados coligidos neste trabalho permitiram concluir acerca da importância do regime de fluxo, sob o qual os biofilmes são formados, na susceptibilidade destes aos produtos antimicrobianos, bem como, no sucesso/falibilidade dos procedimentos de controlo de biofilmes usando esses agentes antimicrobianos, fundamentalmente quando condições extremas são testadas. As bactérias desenvolvidas em biofilme, após tratamento com os agentes químicos, demonstraram recuperar, num curto espaço de tempo, a sua actividade metabólica e viabilidade, mesmo quando os biofilmes apresentavam actividade respiratória residual. Esta constatação é indício irrefutável da capacidade de recrescimento dos biofilmes após o interregno da aplicação dos agentes antimicrobianos. A submissão de biofilmes previamente expostos aos agentes antimicrobianos, a condições hidrodinâmicas diferentes das quais foram desenvolvidos não conduziu à erradicação total dos biofilmes das superfícies. Todos os factos anteriores podem conduzir ao aparecimento de biofilmes recalcitrantes.

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A_0 – Respiratory activity without chemical treatment

A_1 – Respiratory activity immediately after the application of each chemical concentration

APS – Ammonium persulfate

ASI – American steel institute

ATCC – American type culture collection

ATP – Adenosine triphosphate

BCA – Bicinchoninic acid

BSA – Bovine serum albumin

BSE – Bovine spongiform encephalopathy

BOM – Biological oxygen monitor

CFU – Colony forming units

CIP – Clean-in-place

CJD – Creutzfeldt-Jakob disease

CMC – Critical micellar concentration

CRA – Chlorine-releasing agent

CTC – 5-cyano-2, 3-ditolyl tetrazolium chloride

CTAB – Cetyltrimethyl ammonium bromide

DAPI – 4`, 6 – diamidino – 2 – phenylindole

DNA – Deoxyribonucleic acid

d`NTP – deoxyribonucleoside triphosphate

DO- Dissolved oxygen

EPS- Extracellular polymeric substances

GTA - Glutaraldehyde

HACCP – Hazard analysis critical control points

HBV – Hepatitis B virus

HIV - Human immunodeficiency virus

HSL – Homoserine lactones

INT – 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride

L/D – Live/Dead® *Bac*Light Bacterial viability kit

MBC – Minimum bactericidal concentration

Mdr – Multidrug resistance

NaOH – Sodium hydroxide

$N`Re_A$ – Reynolds number of agitation

OD – Optical density

OMP – Outer membrane proteins

OPA – *ortho*-phthalaldehyde

P – Statistical significance level

PCA – Plate Count Agar

PCR – Polymerase chain reaction

PI – Propidium iodide

QAC – Quaternary ammonium compound

RNA – Ribonucleic acid

RLU – Relative light units

rpm – Rotations *per* minute

SEM – Scanning electron microscopy

SD – Standard deviation

SDS – Sodium dodecyl sulfate

SDS – PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SHC – Sodium hypochlorite

Surfactant – Surface active agent

TVS – Total volatile solids

V – Volume

VBNC – Viable but not culturable

W – Weight

W_0 – Biofilm mass without chemical treatment

W_1 – Biofilm mass after chemical treatment

XTT – Benzenesulfonic acid hydrate

Chapter 1

Work outline

Abstract

This chapter provides a general framing of this thesis, working as a guide line to the overall works presented in the further chapters.

1.1 Work outline

Biofilm science is a relatively new technical discipline, which has emerged in response to the need of methodologies for biofilm control. Biofilms represent an interdisciplinary research area focused on the understanding and modulating of the combination of biological and chemical reactions, as well as in the transport and interfacial transfer processes, that potentially affect the microbial accumulation and activity on surfaces. Research on biofilms has progressed rapidly in the last decade. Due to the fact that biofilms have required the development of new analytical tools, many recent advances have resulted from collaborations between microbial ecologists, environmental engineers and mathematicians. The scientific community has come to understand many things about the particular biology of microbial biofilms through a variety of microscopic, physical, chemical, and molecular techniques of study.

This work comes on the sequence of two PhD thesis (Vieira, 1995; Pereira, 2001) developed at the Department of Biological Engineering, University of Minho, where biofilms were characterized and attempts for biofilm control were developed. Probably, the final conjugation of the three PhD thesis can be seen as a Biofilm book constituted by three issues, where aspects from biofilm formation to biofilm control are fully broached. The main goal of the investigation behind this thesis was to characterize biofilms formed under experimental conditions (hydrodynamic conditions, reactor type, strain, etc) that mimic conditions found in actual environments, and to characterize the behaviour of those biofilms when challenged by different chemical agents (non-oxidizing biocides, surfactants, oxidizing biocides).

The microorganism used throughout this work was the Gram-negative bacteria *Pseudomonas fluorescens*. A strain from the American type culture collection (ATCC) and two strains isolated from a process in dairy industry were selected for the overall studies. The use of this bacterium, as model microorganism, is related to the fact that it is ubiquitous in biofilms formed in industrial systems and has potential to cause serious problems in terms of process and final product safety in food industry (Vieira, 1995; Pereira, 2001; Pereira and Vieira, 2001; Dogan and Boor, 2003). The availability of information regarding the growth conditions and biofilm formation properties and behaviour (Oliveira *et al.,* 1994; Vieira, 1995; Pereira, 2001) was also a decisive factor behind that choice.

This thesis is divided in eleven chapters:

Chapter 1 shows the main objectives, context and motivations for the development of this work.

Chapter 2 encloses the literature review, where are described the phenomenon carried on from biofilm formation to biofilm control. Some technical aspects previously focused in the works made by Vieira (1995) and by Pereira (2001) are only mentioned in this chapter, being described in more detail the relatively new aspects involving biofilm formation and control. This literature review gives special attention to the occurrence of biofilms in the food industry, more precisely in the dairy industry. Concerning biofilm control, it is focused on the chemical control of biofilms, being the mechanisms of microbial resistance to antimicrobial agents described in detail, taking into account the literature existent and the relevance of this subject on the understanding of the work presented subsequently.

In Chapter 3, are fully described the materials and methodologies used to perform all the experimental work. Therefore, this chapter is closely linked with every chapter related with experimental work since in these chapters the materials and methods used are briefly referred.

Chapter 4 provides a three methods (short-term bacterial respiratory activity measurement based on the rate of oxygen uptake needed to oxidise glucose; determination of viable and nonviable cells using Live/Dead® *Bac*Light™ kit; colony formation units) comparative study in order ascertain the reliability of respiratory activity as an indicator of the antimicrobial efficacy of *ortho*-phthalaldehyde (OPA) against *P. fluorescens*.

Chapter 5 concerns the characterization of bacteria in planktonic and biofilm state. With this experimental work, it is possible to better understand the bacterial phenotypic changes due to biofilm formation in a flow reactor system, in order to full characterize the cells before being treated with the antimicrobial agents. The influence of the hydrodynamic conditions in biofilm characteristics are evaluated as well as their effects in the cellular phenotype.

Several antimicrobial agents are tested against planktonic cells before being tested against cells within biofilms, being this data present in Chapter 6. The antimicrobial properties of several chemicals and an attempt to discover more about the mechanisms of action of two aldehyde-based biocides - OPA and glutaraldehyde (GTA) - and two surfactants - cetyltrimethyl ammonium bromide (CTAB) and sodium dodecyl sulfate (SDS) are also shown in this chapter. The efficacy of the antimicrobial agents is assessed in

conditions that mimic disinfection in industrial systems under dirty conditions, according to the European Standard EN 1276 (1997).

The effectiveness of OPA and GTA to control biofilms formed under turbulent and laminar flow is compared in Chapter 7. The strategy of biocide application (increase concentration or increase contact time) is determined. The biofilm recovery/regrowth after chemical treatment or a sustained post-biocide application effect is ascertained 3 h after treatment.

In Chapter 8 is presented a study about the comparative action of CTAB and SDS in the control of biofilms formed under turbulent and laminar flow, as well as an attempt to understand the mode of action of both surfactants against the biofilms. In this chapter the emerge of biofilms able to recover their metabolic characteristics and viability after surfactant treatment or a sustained post-surfactant application effect is also assessed.

In Chapter 9, a bioreactor with an immersed rotating device is used to form biofilms and to assess biofilm mechanical stability before and after chemical treatment. Therefore, the biofilms are characterized and the synergistic action of chemical and mechanical treatment is assessed as a mean to remove biofilms. Two aldehyde-based biocides (OPA and GTA), two surfactants (CTAB and SDS) and two oxidizing-biocides (sodium hydroxide and hypochlorite chloride) are tested.

In Chapter 10 the ability of two *P. fluorescens* strains isolated from an industrial process to form biofilms in the flow cell reactor, under turbulent and laminar flow, and in the bioreactor with the immersed rotating device is characterized. The phenotypic characterization as a consequence of biofilm formation under different hydrodynamic conditions, reactor design and community interactions (single/mixed biofilms) is assessed.

Chapter 11 gives an overview of the work presented and identifies future research to advance the optimization of methods to control unwanted biofilms.

Chapter 2

Literature review - from biofilm formation to biofilm control

Abstract

This chapter reviews current knowledge on the features of biofilm formation and control, with a special focus on the control and resistance of biofilms face to chemical agents.

2.1 Biofilms – the discover of a new microbial arrangement

It is a natural tendency of microorganisms to attach to surfaces, to multiply and to embed themselves in a slimy matrix, resulting in biofilms. These allow complex interactions among different species. It is supposed that biofilm is the first form of life recorded on the planet, being estimated that most microorganisms on the earth are organized in biofilms and they even occur in extreme environments such as hydrothermal vents, nuclear power plants and disinfection pipelines (Costerton *et al.,* 1987).

Concerning the discover of biofilms, it as been first documented in 1943 by Zobell, where were observed microbial cells attached in layers to bottle walls and that the addition of glass rods increased the biological activity of batch suspended cultures. Latter, Atkinson and co-workers (1964, 1967) coined the term microbial or biological film to represent the gelatinous layer of cells and their adherent by-products on bioreactor vessel walls. Topiwala and Hamer (1971) and Howell *et al.* (1972) referred to mucilaginous layers of bacterial cells and their extracellular polymeric substances as "wall growth". Characklis (1973a, b) provided an extensive two-part literature review on the basic fundamentals and practical implications of "microbial slimes". Atkinson (1964) and Atkinson and co-workers (1967) and Harremoës (1977) applied heterogeneous catalyst mathematics to describe simultaneous mass transport and biological reaction within "microbial films". A consensus of the leaders in biofilm research in 1984 termed a biofilm as a collection of microorganisms, predominantly bacteria, enmeshed within a three-dimension gelatinous matrix of extracellular polymers secreted by the microorganisms (Marshall, 1984).

2.2 Processes governing biofilm formation and performance

Initially, the adhesion surface is conditioned and cells attach first reversibly, and then irreversibly. Next, attached cells grow, reproduce, and secrete insoluble extracellular substances. As the biofilm matures, biofilm detachment and growth processes come into balance, such that the total amount of biomass on the surface remains approximately constant in time.

At the time, processes governing biofilm formation and persistence (Figure 2.1) included:

1. Preconditioning of the adhesion surface either by macromolecules present in the bulk liquid or intentionally coated;
2. Transport of planktonic cells from the bulk liquid to the surface;
3. Adsorption of cells at the surface for a finite time;
4. Desorption (release) of reversible adsorbed cells;
5. Irreversible adsorption of bacterial cells at a surface;
6. Transport of substrates to and within the biofilm;
7. Substrate metabolism by the biofilm-bound cells and transport of products out of the biofilm. These processes are accompanied by cellular growth, replication, and extracellular polymeric substances production;
8. Biofilm removal by detachment or sloughing.

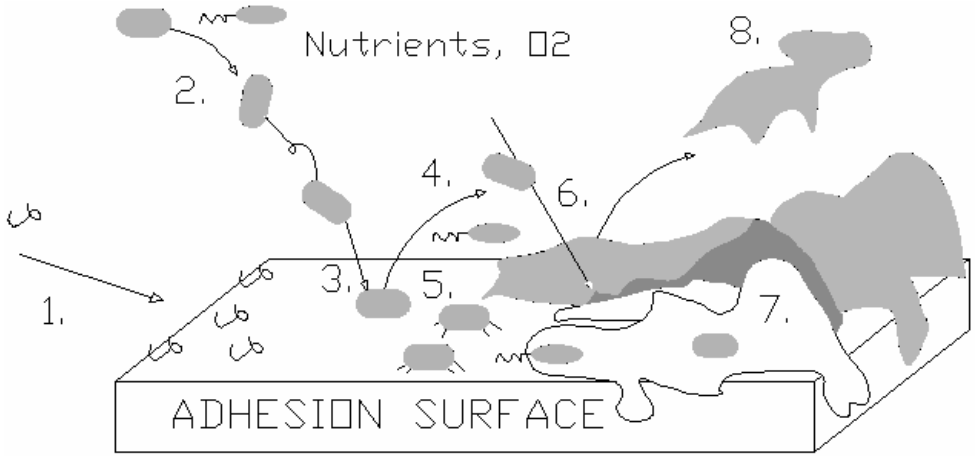


Figure 2.1 Processes governing biofilm formation (based on Bryers, 2000).

Research in the last ten years has expanded the understanding of the molecular and genetic parameters that control many of these macroscopic processes. Biofilms (Figure 2.2) are no longer considered uniform biological structures in time and space, and processes that control this heterogeneity have been characterized and are being mathematically described (Bryers, 2000).

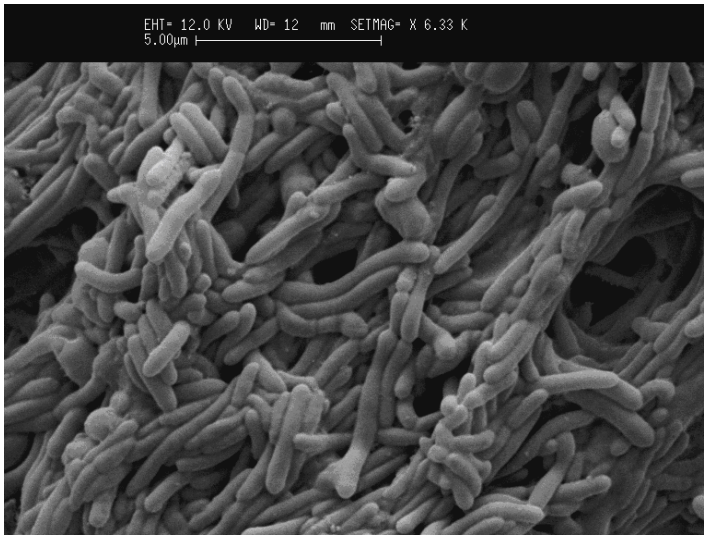


Figure 2.2 Scanning electron microscopy photomicrographs of a 6 d old *Bacillus cereus* biofilm formed on stainless steel slides. X 6330 magnification; bar = 5 µm.

2.3 Parameters affecting biofilm formation

There exist a number of mechanisms by which numbers of species of microorganisms are able to come into closer contact with a surface, attach firmly to it, promotes cell-cell interactions and grow as a complex structure (Bryers, 2000).

The attachment of microorganisms to surfaces is a very complex process, with many variables affecting the process. In general, attachment will occur most readily on surfaces that are rougher, more hydrophobic, and coated by surface conditioning films (Pereira, 2001; Donlan, 2002). An increase in flow velocity, water temperature, or nutrient concentration may also be equated to increase attachment, if these factors do not exceed critical levels (Vieira, 1995; Pereira, 2001). Properties of the cell surface, specially the presence of extracellular appendages, the interactions involved in cell-to-cell communication and the production by the microorganisms of extracellular polymeric substances are important factors that may possibly provide a competitive advantage for one microorganism where a mixed community is involved (Donlan, 2002).

Table 2.1 summarizes the main variables involved in cell attachment and biofilm formation.

Table 2.1 Variables important in cell attachment, biofilm formation and development (based on Donlan, 2002)

Properties of the adhesion surface	Properties of the bulk fluid	Properties of the cell
	Flow velocity	Cell surface hydrophobicity
Texture or roughness	pH	Extracellular appendages
Hydrophobicity	Temperature	Extracellular polymeric
Conditioning film	Ions	substances
	Presence of antimicrobial agents	Signalling molecules

The aspects of biological attribute, *i.e.,* the aspects dependent on the cell characteristics, will be ahead described in detail in the following sections.

2.3.1 Specialized attachment structures/surface properties of the cell

Cell surface hydrophobicity and the presence of extracellular filamentous appendages may influence the rate and the extent of attachment of microbial cells. The hydrophobicity of the cell surface is important in adhesion because hydrophobic interactions tend to increase with an increasing nonpolar nature of one or both surfaces involved, *i.e.,* the microbial cell and the adhesion surface (Donlan, 2002). According to Drenkard and Ausubel (2002), the ability of bacteria to attach to each other and to surfaces depends in part on the interaction of hydrophobic domains.

Many cells produce extracellular filamentous appendages. These may, therefore, play a role in the attachment process, seeing as their radius of interaction with the surface is far lower than that of the cell itself. A number of such structures are known to exist - *flagella*, *pili* or *fimbrae*, *prothecae*, *stalks* and *holdfast* (Harbron and Kent, 1988).

Flagella, when existent, are responsible for the motility of bacteria. These are very fine threads of the protein flagellin with a helical structure extending out from the cytoplasm through the cell wall. *Flagella* may have a diameter between 0.01 to 0.02 μm, and a length of up to 10 μm. Many types of bacteria have *flagella*, including the genus *Pseudomonas*. It is possible that the flagellum itself may form an adhesive bond with the adhesion surface (Harbron and Kent, 1988). The primary function of *flagella* in biofilm formation is assumed to be in transport and in initial cell-to-surface interactions (Sauer and Camper, 2001).

Flagella-mediated motility is believed to overcome repulsive forces at the surface of the substratum and, as a consequence, a monolayer of cells forms on the adhesion surface (Daniels *et al.,* 2004).

Pili or *fimbriae* are found on many Gram-negative bacteria including *Pseudomonas* species. They are fine, filamentous appendages, also of protein, 4 to 35 nm wide and up to several micrometers long. These structures are usually straight, and are not involved in motility. Their only known general function is to make cell more adhesive, since bacteria with *pili* can adhere strongly to other bacterial cells, and inorganic particles (Rogers, 1979). Nevertheless, they are not always involved in the attachment process even if they are present (Characklis and Cooksey, 1983). According to Sauer and Camper (2001), *Pili* and pilus-associated structures have been shown to be important for the adherence to and colonization of surfaces, probably by overcoming the initial electrostatic repulsion barrier that exists between the cell and the substratum (Donlan, 2002).

Prosthecae and *stalks* form a third group of attachment structures. These occur in several types of microorganisms including such Gram-negative bacteria as *Caulobacter* and *Hyphomicrobium*. They may occur at one or more sites on the surface of the cell, and are filiform or blunt extensions of the cell wall and membrane, commonly 0.2 μm (Harbron and Kent, 1988). At the end of a *prosthecae* or *stalk* is usually found an adhesive disk, or *hold-fast* (Rogers, 1979). The *stalk* and *hold-fast* structure is one quite often used by diatoms to attach to a surface (Harbron and Kent, 1988).

2.3.2 Extracellular polymeric substances

2.3.2.1 Extracellular polymeric substances – definition, composition, secretion and function

Extracellular polymeric substances (EPS) were defined by Geesey (1982) as extracellular polymeric substances of biological origin that participate in the formation of microbial aggregates (Figure 2.3). In biofilm systems, EPS are responsible for binding cells and other particulate materials together - cohesion and to the surface - adhesion (Characklis and Wilderer, 1989; Sutherland, 2001; Allison, 2003). The general composition of bacterial EPS comprises polysaccharides, proteins, nucleic acids, lipids, phospholipids, and humic substances, since the last ones are sometimes considered as part of the EPS matrix (Jahn and

Nielsen, 1998; Wingender *et al.,* 1999; Sutherland, 2001). In contrast to microbial capsules, EPS are exudates which are not firmly bound to the cell surface.

Biofilms form a gel phase where microorganisms live inside (Wingender *et al.,* 1999; Sutherland, 2001). The EPS matrix acts as a barrier in which diffusive transport prevails over convective transport (Sutherland, 2001). However, this matrix is not uniform and homogeneous – pores, channels and areas with EPS of low density can occur (Sutherland, 2001; Fux *et al.,* 2005a). Thus, diffusion coefficients within a biofilm do not differ too profoundly from those in free water; only large molecules diffuse significantly slower in a biofilm (Flemming, 1996; Dignac *et al.,* 1998; Wingender *et al.,* 1999). The presence of charged groups such as pyruvate or uronic acids influences their physical properties (stability, viscosity) and provides ion exchange sites (Flemming, 1996; Higgins and Novak, 1997). EPS are hydrophilic (Wingender *et al.,* 1999). If a hydrophobic surface is colonized, the EPS represent a hydrophilic interface thus masking the original surface properties.

By definition, EPS are located at or outside the cell surface independent of their origin. The extracellular localization of EPS and their composition may be the result of different processes: active secretion, shedding of cell surface material, cell lysis, and adsorption from the environment (Wingender *et al.,* 1999). Various specific pathways of biosynthesis and discrete export mechanisms involving the translocation of EPS across bacterial membranes to the cell surface or into the surrounding medium have been described for bacterial proteins and polysaccharides (Becker *et al.,* 1998; Hueck *et al.,* 1998). Extracellular deoxyribonucleic acids (DNA) can be produced by bacteria during growth, being proposed by Lorenz and Wackernagel (1994), that DNA can be actively secreted or passively released due to increase in cell envelope permeability. Another mechanism of release of extracellular polymers is the spontaneous liberation of integral cellular components from the outer membrane of Gram-negative bacteria, due to the formation of outer membrane derived vesicles which has been described by Beveridge *et al.* (1997) as a common secretion mechanism. The release of cellular material by this mechanism may be the result of metabolic turnover processes, since it occurs during normal growth (Beveridge *et al.,* 1997). Death and lysis of cells contribute to the release of cellular high-molecular-weight compounds into the medium and entrapment with the biofilm matrix, a process where the biofilm represents a recycling yard for intracellular components (Wingender *et al.,* 1999).

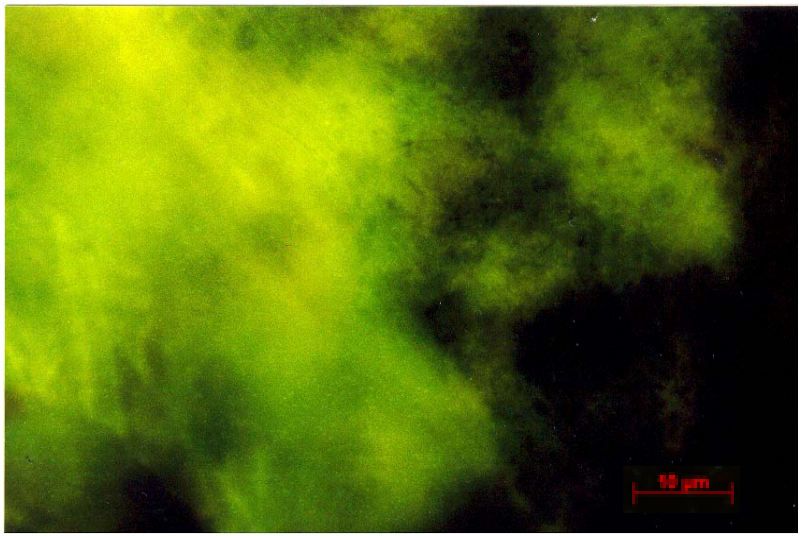


Figure 2.3 Epifluorescence photomicrograph of a 6 d old *P. fluorescens* biofilm formed on stainless steel slides and stained with acridine orange at 0.003 % (w/v). X 100 magnification; bar = 10 µm; green colour – extracellular polymeric matrix; black colour – background.

The properties of the community of microbial cells within the EPS matrix are greater than that of the sum of the individual microorganisms (Sutherland, 2001). One of the most important functions of EPS is supposed to be their role as fundamental structural elements of the biofilm matrix determining the mechanical stability of biofilms, mediated by non-covalent interactions either directly between the polysaccharide chains or indirectly via multivalent cation bridges (Flemming 1996; Allison, 2003). Higgins and Novak (1997) suggest that lectin-like proteins also contribute to the formation of the three-dimensional network of the biofilm matrix by cross-linking polysaccharides directly or indirectly through multivalent cation bridges. Among activated sludge extracellular polymers, proteins predominated and, on the basis of their relatively high content of negatively charged amino acids they were supposed to be more involved than sugars in electrostatic bonds with multivalent cations, underlining their key role in the floc structure (Dignac *et al.,* 1998). In addition, proteins have also been suggested to be involved in hydrophobic bonds within the EPS matrix (Dignac *et al.,* 1998). Nevertheless, the main function of extracellular proteins in biofilms is mostly seen in their role as enzymes performing the digestion of exogenous macromolecules and particulate material in the microenvironment of the immobilized cells. Thus, they provide low molecular weight nutrients which can readily be taken up and metabolized by the cells (Junter *et al.,* 2002). Enzymes within the biofilm matrix may also

be involved in the degradation of polysaccharidic EPS causing the release of biofilm bacteria and the spreading of the microorganisms to new environments (Boyd and Chakrabarty, 1994; Wingender *et al.,* 1999).

A function frequently attributed to EPS is their general protective effect on biofilm organisms against adverse conditions. As an example, it has frequently been observed that biofilm cells can tolerate significantly higher concentrations of biocides (Foley and Gilbert, 1996; Mah and O´Toole, 2001; Pereira and Vieira, 2001). This is supposed to be due mainly to physiological changes of biofilm bacteria enhancing their resistance to biocides, but also to a barrier of EPS (Morton *et al.,* 1998; Pereira and Vieira, 2001). The EPS matrix delays or prevents biocides from reaching target microorganisms within the biofilm by diffusion limitation and/or chemical interaction with the EPS molecules (Heinzel, 1998; Mah and O´Toole, 2001; Pereira and Vieira, 2001; Gilbert *et al.,* 2002).

The role of EPS components other than polysaccharides and proteins remains to be established (Wingender *et al.,* 1999). Nevertheless, it is expected that EPS such as lipids and nucleic acids significantly influence the rheological properties and thus the stability of biofilms (Neu, 1996). Concerning the extracellular DNA, Whitchurch *et al.* (2002) found that it is required for the initial establishment of biofilms by *P. aeruginosa*, and possibly for biofilms formed by other bacteria that specifically release DNA. Moreover, within the matrix formed by the EPS, the molecules required for cell-cell communication and community behaviour might accumulate at high enough concentrations to be effective (Sutherland, 2001).

Table 2.2 Effects associated to EPS formation in biofilms (based on Wingender *et al.,* 1999)

Function	Relevance
Adhesion to surfaces	Initial step in the colonization of inert and tissue surface, accumulation of bacteria on nutrient-rich surfaces in oligotrophic environments
Aggregation of bacterial cells, formation of flocs and biofilms	Bridging between cells and inorganic particles trapped from the environment, immobilization of mixed bacterial populations, basis for development of high cell densities, generation of a medium for communication processes, cause for biofouling and biocorrosion events
Cell-cell recognition	Symbiotic relationships with plants or animals, initiation of pathogenic processes
Enzymatic activities	Digestion of exogenous macromolecules for nutrient acquisition, release of biofilm cells by degradation of structural EPS of the biofilm
Interaction of polysaccharides with enzymes	Accumulation/retention and stabilization of secreted enzymes
Protective barrier	Resistance to non-specific and specific host defences, resistance to biocides
Sorption of exogenous organic compounds	Scavenging and accumulation of nutrients from the environment
Sorption of inorganic ions	Accumulation of toxic metal ions, promotion of polysaccharide gel formation and mineral formation
Structural elements of biofilms	Mediation of mechanical stability of biofilms, determination of the shape of EPS structure.

2.3.3 Cell communication (quorum sensing)

The concept of bacterial development, organization and evolution as communities comprises the existence of self-organization and cooperativity among cells as a driving force in community development, rather than the classical natural selection of individual microorganisms (Davies *et al.,* 1998; Fuqua and Greenberg, 2002; Daniels *et al.,* 2004; Fux *et al.,* 2005a; Parsek and Greenberg, 2005). This concept becomes specially apparent when

examining bacterial biofilm communities (Decho, 1999; Parsek and Greenberg, 2005). Cell-to-cell signalling has recently been demonstrated to play a role in cell attachment and detachment from biofilms (Donlan, 2002; Daniels *et al.,* 2005). Bacteria are considered to be far from solitary microorganisms, rather being colonial by nature and exploiting elaborate systems of intercellular interactions and communications to facilitate their adaptation to changing environments (Bainton *et al.,* 1992; Davies *et al.,* 1998; Decho, 1999; Sauer and Camper, 2001; Fuqua and Greenberg, 2002; Daniels *et al.,* 2004). The successful adaptation of bacteria to changing natural conditions requires that the microorganism is able to sense and respond to its external environment and modulate gene expression accordingly (Decho, 1999; Daniels *et al.,* 2004). Quorum sensing is based on the process of autoinduction (Fuqua *et al.,* 1994; Daniels *et al.,* 2004). The process of quorum sensing provides a mechanism for self-organization and self-regulation by microbial cells (Decho, 1999; Parsek and Greenberg, 2005). It involves an environmental sensing system that allows bacteria to monitor and respond to their own population densities. The bacteria produce a diffusible organic signal, originally called an autoinducer molecule, which accumulates in the surrounding environment during growth (Fuqua and Greenberg, 2002). High cell densities result in high concentrations of signal, and induce expression of certain genes and/or physiological changes in neighbouring cells (Fuqua *et al.,* 1996; Parsek and Greenberg, 2005). Responses to chemical signals in the process of cell communication is a concentration dependent process, where a critical threshold concentration of the signal molecule must be reached before a physiological response will be elicited (Decho, 1999; Fuqua and Greenberg, 2002; Daniels *et al.,* 2004).

Molecules derived from homoserine lactones (HSL) act as signals in Gram-negative bacteria (Eberhard *et al.,* 1981; Fuqua and Greenberg, 2002; Parsek and Greenberg, 2005). Acyl-HSL`s have been identified in many different Proteobacteria (Fuqua and Greenberg, 2002). These signal molecules are composed of a fatty acyl chain bonded to a lactonized homoserine through an amide bond. There is considerable structural variety between acyl-HSL`s from different bacteria and even between different acyl-HSL`s synthesized by the same bacteria (Fuqua and Greenberg, 2002; Parsek and Greenberg, 2005). Although many different N-acyl homoserine lactone autoindutors have been isolated from various Gram-negative bacteria, all differences are in the N-acyl side chain length (C_4 to C_{14}) or degree of substitution - either 3-oxo, 3-hydroxy, saturated, or unsaturated (Pearson *et al.,* 1999; Fuqua and Greenberg, 2002).

Quorum sensing systems are known to be involved in a range of important microbial activities. These include extracellular enzyme biosynthesis, biofilm development, antibiotic biosynthesis, biosurfactant production, extracellular polymeric substances synthesis and extracellular virulence factors in Gram-negative bacteria (Passador *et al.,* 1993; Beck von Bodman and Farrand, 1995; Chatterjee *et al.,* 1995; McGowan *et al.,* 1995; Pearson *et al.,* 1995; Davies *et al.,* 1998; Daniels *et al.,* 2004; Fux *et al.,* 2005a).

2.4 The impact of biofilm formation

Biofilms are as versatile as they are ubiquitous. Intentional and unintentional biofilms concern a broad range of areas, comprising special attention in the industrial/environmental and biomedical areas (Bryers, 2000).

2.4.1 Beneficial biofilms

Benefits afforded by biofilms in a continuous reactor situation arise chiefly because the cell population is immobilized and thus the residence time of cells in the reactor is independent of the fluid phase residence time. In continuous suspended culture bioreactors, the mean residence time of the system cannot be less than the generation time of the bacterial specie, otherwise cells do not have sufficient time to replicate within the reactor and are eventually diluted from the system (Pereira, 2001; Junter *et al.,* 2002).

One major application that relies on a microbial culture ability to form biofilms is waste-water treatment. Biofilm reactor configurations, applied in both pilot and full-scale waste-water treatment, include packed bed (trickling filters), high rate plastic media filters, rotating biological contactors, fluidized-bed biofilm reactors, and membrane immobilized cell reactors (Pereira, 2001; van Loosdrecht *et al.,* 2002).

Immobilized and biofilm-bound cells remain in a continuous reactor system independent of the fluid phase, thus the mass loading of limiting substrate or influent pollutant in the case of a wastewater treatment reactor can be increased well beyond the growth rate limit imposed on suspended cultures (Junter *et al.,* 2002; van Loosdrecht *et al.,* 2002). Consequently, immobilized-cell or biofilm reactors can provide added volumetric reactivity, more stable operating performance and an inherent ease in biomass - fluid separation; the prospect of staging different bioconversion processes in sequential reactors

(Pereira, 2001). Due to these inherent advantages, the use of biofilm reactors is not confined only to bacterial cells, but also comprises plant and animal cell applications (Junter *et al.,* 2002). Bacterial biofilm reactors are employed either in commodities production or in waste-water treatment applications. Biofilm reactors have been reportedly used to produce organic compounds with human use (Park and Toda, 1992; Kwak and Rhee, 1992; Velizarov *et al.,* 1992).

From an ecological point of view, life in a biofilm may offer important advantages to the cells (Flemming and Schaule, 1996).

Table 2.3 summarizes the probable benefits of biofilm growth for microorganisms.

Table 2.3 Benefits of biofilm growth for microorganisms (according to Davey and O´Toole, 2000)

Create their own microniche;
Excretion of carbon compounds under limitation of other growth factors;
Increased absorption of nutrients by EPS;
Increased availability of nutrients by uptake from liquid passing by;
Interspecies support within nutritional chains, interspecies communication;
Protection against desiccation.

2.4.2 Detrimental biofilms

Unintentionally formed biofilms can create such detriments as biofouling of heat exchange systems and marine structures; microbial induced corrosion of metal surfaces or the deterioration of dental surfaces; contamination of household products, food preparations and pharmaceuticals; and the infection of short and long term indwelling biomedical implants and devices. Such detriments can range in severity from being a mere nuisance to being life threatening (Marshall, 1984; Bryers, 1991; Gilbert *et al.,* 2003; McBain *et al.,* 2003; Hall-Stoodley *et al.,* 2004).

Table 2.4 Detrimental effects of biofilm processes (according to Srinivasan *et al.,* 1995)

System	Effects
Cooling water towers and heat exchangers	Energy losses due to increased fluid frictional and heat transfer resistances
Drinking water distribution	Increased suspended solids; coliform contamination
Secondary oil recovery	Plugging of water injection wells corrosion
Process equipment	Material corrosion or biodeterioration
Food processing	Contamination
Metalworking	Degradation of metal working fluid
Paper manufacture	Degradation of product quality
Dental plaque	Caries; periodontal disease
Medical implants, catheters	Persistent infections
Ship hulls	Increased frictional drag
Reverse osmosis membranes	Reduced permeability; material degradation
Clean surfaces (health care, consumer)	Health risks; cosmetic degradation
Swimming pools	Health risks; cosmetic degradation

2.5 Implications of biofilms in food industry

The general aims for microbial control in the food industry, including biofilm removal, are to prevent spoilage of products and to ensure that the quality specifications of the product are met. The most important means for maintaining efficient microbial control include (Figure 2.4): minimizing the microbial load from outside sources to the process; efficient control of growth at microbiologically vulnerable sites; adequate cleaning and disinfection of the process lines (Wirtanen *et al.,* 2000).

The target of microbial control in a process line is two-fold: to reduce or limit the number of microbes and their activity and to prevent and control the formation of deposits on process equipment. Nowadays, the most efficient means for limiting the growth of microbes are: good production hygiene, a rational running of the process line, and as well designed use of biocides and disinfectants (Maukonen *et al.,* 2003). Novel means to control biofilm formation are constantly sought through the control of environmental factors, on the process line and the use of surfactants, biosurfactants, enzymes, and new antimicrobial

agents. The cleaning and disinfection of surfaces, the training of personnel and good manufacturing and design practices are important in combating hygiene problems in food industry (Wirtanen *et al.,* 2000; Maukonen *et al.,* 2003). Disinfection after the removal of biofilms, using suitable cleaning procedures, is also required in food plants where wet surfaces provide favourable conditions for microbial growth (Carpentier and Cerf, 1993)

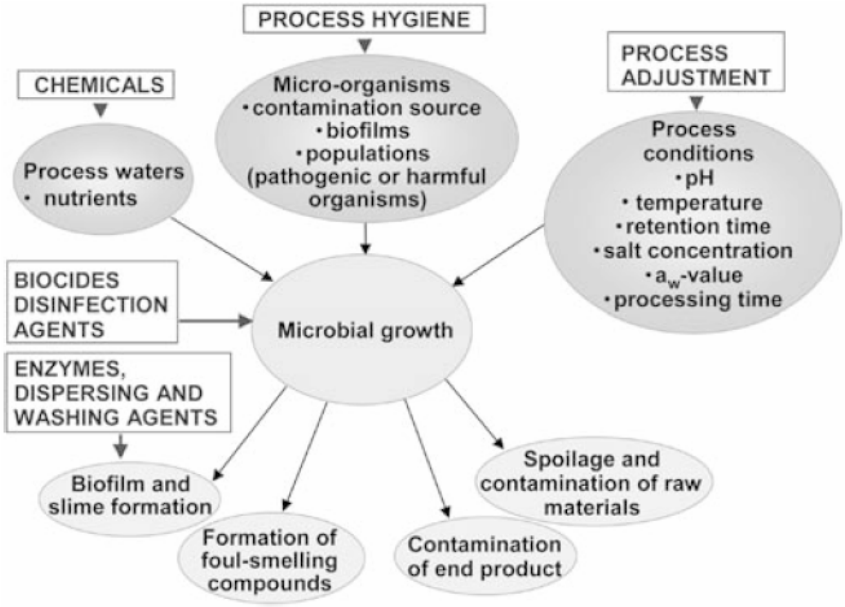


Figure 2.4 Sources, problems, and control of microbial contaminants in industrial processes (according to Maukonen *et al.,* 2003).

The attachment of the bacteria to the food product or the product contact surfaces leads to serious hygienic problems and economic losses due to food spoilage (Carpentier and Cerf, 1993; Wirtanen *et al.,* 2000; Maukonen *et al.,* 2003). In addition to that, a number of reports have appeared on the persistence of several foodborne pathogens on food contact surfaces (Kumar and Anand, 1998). If pathogens are present, then consumption of the contaminated product may pose a health risk (Chmielewski and Frank, 2003). Commonly found microorganisms in the food industry and on food contact surfaces are enterobacteria, lactic acid bacteria, micrococci, streptococci, pseudomonads, and bacilli (Maukonen *et al.,* 2003). *Pseudomonas* spp. are important bacterial contributors to spoilage of conventionally pasteurized fluid milk products, acting by two different ways. First, they produce the majority of lipolytic and proteolytic enzymes secreted into raw milk during pre-processing storage. Many of these enzymes can survive pasteurization (72 ºC for 15 s) and even ultra-high-temperature treatments (138 ºC for 2 s or 149 ºC for 10 s) and can thus reduce the

sensory quality and shelf life of the processed fluid milk products. Second, *Pseudomonas* spp. can act in the post-pasteurization process, causing spoilage of conventionally pasteurized milk during refrigerated storage (Wiedmann *et al.,* 2000; Dogan and Boor, 2003).

In the dairy industry, improperly cleaned and disinfected equipment and air-borne microorganisms are usually considered to be the main sources of contamination of milk and milk products. Wong (1998) reported that undesirable microorganisms such as *Lactobacillus curvatus* and *Lactobacillus fermentum* persisted on milk residues in cheese processing plants even after repeated cleaning, subsequently contaminating products. Processing environments commonly contribute to post-pasteurization contamination of pasteurized milk products, specifically, filling machines are an important source of contamination of pasteurized milk (Dogan and Boor, 2003). Some microorganisms, in biofilms, catalyze chemical and biological reactions causing corrosion of metal in pipelines and tanks (Chmielewski and Frank, 2003). Reduction in the efficacy of heat transfer (Mittelman, 1998) can occur if biofilms become sufficiently thick at locations such as plate heat exchangers.

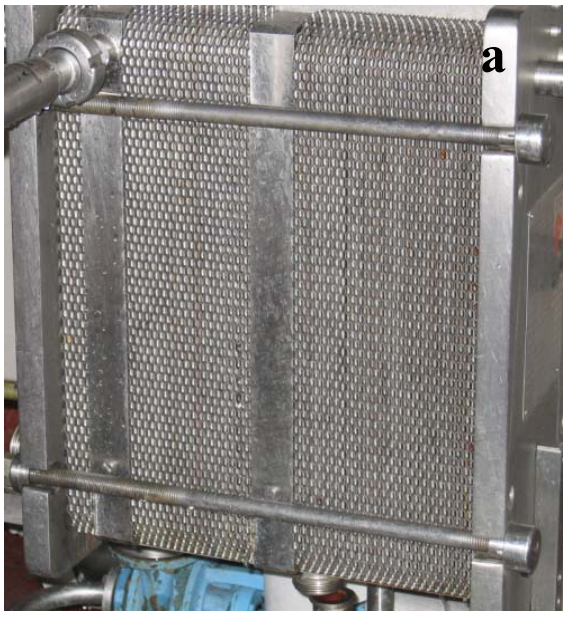
The time available for biofilm formation will depend on the frequency of cleaning regimes. Product contact surfaces may typically be cleaned several times *per* day, while environmental surfaces such as walls may be cleaned once *per* day (Gibson *et al.,* 1999). There is, therefore, more time for biofilm formation on environmental surfaces. Gibson *et al.* (1995) found that although attachment to a variety of surfaces in the food processing environment readily occurred, extensive surface colonization and biofilm formation only occurred on environmental surfaces. Product contact surfaces may contaminate the product directly as the product touching or passing over the surface will potentially pick up microbial contamination (Gibson *et al.,* 1995). Environmental surfaces such as floor and walls may be indirect sources of microbial contamination that can be transferred to the product by vectors such as the air, personal and cleaning systems (Holah, 1992; Gibson *et al.,* 1999). The hygiene of the surfaces, therefore, affects the quality and safety of the food product.

The key to the effective cleaning and disinfection of food plants is the understanding of the type and nature of the soil (carbohydrates, fat, proteins, mineral salts) and the microorganisms to be removed from the surfaces (Troller, 1993). Furthermore, the selection of detergents and disinfectants in food industry depends on the efficacy, safety and

rinsability of the agent as well as if it is corrosive or affects the sensory values of the product manufactured (Wirtanen *et al.,* 2000).

In food industry, equipment design and choice of surface materials are important in fighting biofilm formation. The most practical material in processing equipment is steel, which can be treated with mechanical grinding, brushing, lapping, and electrolytic or mechanical polishing. Dead ends, corners, cracks, crevices, gaskets, valves and joints are vulnerable points for biofilm accumulation (Maukonen *et al.,* 2003). Provided that the equipment and environment are hygienically designed (with no crevices, dead spaces, surface material, etc), an effective cleaning and disinfection programme is the main method of control of the surface route of contamination. An effective disinfection programme removes undesirable material from the surfaces, including microorganisms, soil, foreign bodies and cleaning chemicals. This involves a number of stages: wetting of the soil and surface by the cleaning chemical; reaction of the chemical agent to facilitate removal from the surface; prevention of re-deposition and disinfection of residual microorganisms (Holah, 1992; Gibson *et al.,* 1999). Cleaning-in-place (CIP) procedures are usually employed in milk processing lines. Nevertheless, the limitation of CIP procedures is the accumulation of microorganisms on the equipment surfaces, resulting in biofilm formation (Kumar and Anand, 1998; Sharma and Anand, 2002), as can be seen in Figure 2.5.

Figure 2.5 b, shows a plate from an heat exchanger after the application of a CIP procedure, comparing with Figure 2.5 c, it is evident the considerable existence of an organic deposit.



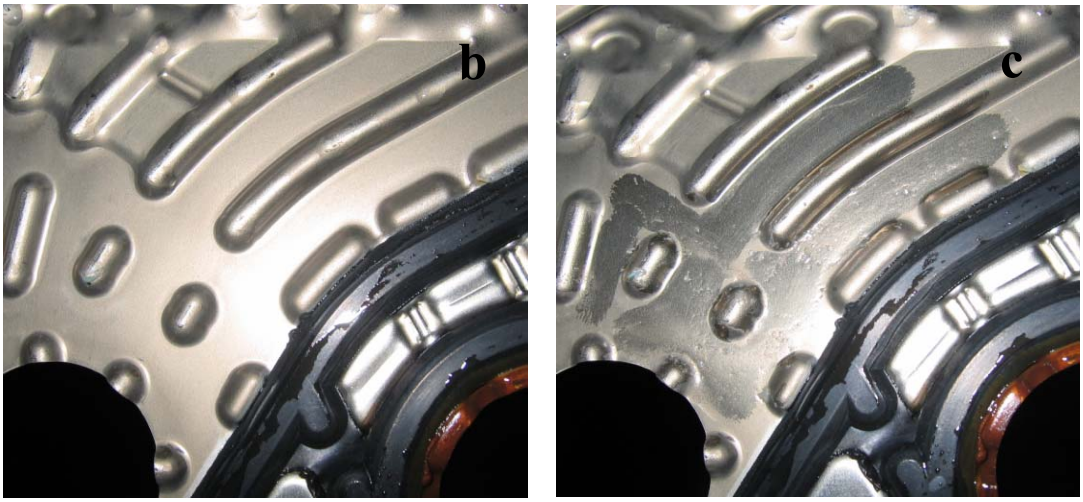


Figure 2.5 Plate heat exchanger (**a**) where plates were removed for analysis of the biological contaminants. Plate before (**b**) and after (**c**) organic deposit removal.

An independent quality control system to monitor the cleaning results for a food plant can be integrated in the **H**azard **A**nalysis **C**ritical **C**ontrol **P**oints (HACCP) program. According to Sharma and Anand (2002), evaluation of biofilms status and development of an effective sanitation plan should be part of the HACCP plan development in order to make them more meaningful. Effective sanitation programmes should be devised based on *in vitro* studies that could be invariably repeated under *in situ* conditions in order to control the biofilms prevalent in dairy/food processing areas (Sharma and Anand, 2002).

2.6 Approach for biofilm mitigation – biofilm prevention

In many technical processes microorganisms are not a problem as long as they remain planktonic. In other fields it would facilitate disinfection if attachment of microorganisms could be prevented. One strategy to prevent the formation of biofilms is to disinfect regularly, before biofilm formation starts (Meyer, 2003). However, there are a number of efforts in order to study strategies to prevent biofilm formation.

Several attempts have been made by Rogers *et al.* (1994) to identify materials that do not promote or even suppress biofilm formation in drinking water distribution systems. The investigators ranked different materials according to biofilm growth of microorganisms in general and *Legionella pneumophila* in particular, concluding that there is hardly that any material does not allow biofilm formation. Nevertheless, such rankings have to be evaluated with caution because they may vary with the microbial species and with the test conditions.

Inhibition of biofilm formation by limitation of the carbon source is a virtually impossible procedure, since ultra-pure water systems have been found to support the formation of biofilms (Griebe and Flemming, 1998). Another approach is to supply the microorganisms with growth factors, so surface attachment is no more a benefit for them (Meyer, 2003).

Several attempts have been made to avoid biofilm formation by incorporation of antimicrobial agents into surface materials or coat surfaces with antimicrobial agents (Meyer, 2003; Thouvenin *et al.,* 2003). This mode of biofilm prevention has more application in the biomedical field. Biofilm formation on implanted medical devices is a frequent cause of implant rejection. Several authors report inhibition of biofilm formation on such devices by coating with silver (Klueh *et al.,* 2000; Hashimoto, 2001). Gottenbos *et al.* (2001) demonstrate a reduction in infection rate using silicone rubber implants with covalently coupled quaternary ammonium coatings. The process of coating materials has yet found its way to broad application. This may be due to the conflicting results regarding the clinical benefit, which are reported by different researchers (Logghe *et al.,* 1997; Berry *et al.,* 2000). In other application fields, like the food industry, possible carry over of antimicrobial agents into food are an only temporary effect, when coatings release antimicrobial agents, restricted the use of these coatings (Meyers, 2003).

Cloete and Jacobs (2001) reported that, surface active agents have been employed to prevent bacterial adhesion to surfaces. Unfortunately, little published information is available on the efficiency of surfactants against bacterial attachment. According to Paul and Jeffrey (1985), dilute surface active agents completely inhibited the attachment of estuarine and marine bacteria.

2.7 New methodologies for biofilms control

The biotechnology sector is just beginning to tackle the problem of biofilms by developing antimicrobial agents with novel mechanisms of action. Some studies seek to prevent biofilm formation, others aim to develop antimicrobial agents to treat existing biofilms, and still others are trying to disrupt the polymeric ties that bind the biofilms together (Schachter, 2003).

2.7.1 Biofilm control with enzymes

The use of enzyme-based detergents as biocleaners, also known as "green chemicals", can serve as a viable option to overcome the biofilm problem in the food industry. However, the technology and production of these enzymes and the enzyme-based detergents is mostly patent-protected. Enzymes can be used to degrade biofilm, but due to the heterogeneity of the EPS in the biofilm, a mixture of enzyme activities may be necessary for a sufficient degradation of bacterial biofilms. Enzymes and detergents have also been used as synergists to improve disinfectant efficacy (Jaquelin *et al.,* 1994; Johansen *et al.,* 1997; Meyer, 2003). The specific mode of action makes it a complex technique, increasing to the difficult that is to find enzymes that are effective against all different types of biofilms. So, formulations containing several different enzymes seem to be fundamental for a successful biofilm control strategy. Basically, proteases and polysaccharide hydrolysing enzymes may be useful (Johansen *et al.,* 1997). Although, the use of enzymes in removal of bacterial biofilms is still limited, partly due to the low prices of the chemicals used today. Also, the low commercial accessibility of different enzyme activities limits their usage (Johansen *et al.,* 1997).

2.7.2 Biofilm control with phages

When phages come in contact with biofilms, further interactions occur, dependent on the susceptibility of the biofilm bacteria to phage and to the availability of receptor sites. If the phage also possesses polysaccharide-degrading enzymes, or if considerable cell lysis is affected by the phage, the integrity of the biofilm may rapidly be destroyed. Hughes *et al.* (1998) working in the control of biofilms of *Enterobacter agglomerans* by the use of phages found that the cells were readily lysed and the biofilm degraded by the addition of bacteriophage if certain criteria were met. The bacteria had to be susceptible to the phage, and the phage polysaccharide depolymerise had to be able to degrade the biofilm EPS. The phage then lysed the biofilm cells, the polymerase enzyme degraded the EPS and caused the biofilm slough off. If only one of these criteria was met, there was still a substantial degree of biofilm degradation. Alternatively, coexistence between phage and host bacteria within the biofilm may be developed (Hughes *et al.*, 1998).

However, phage have been proposed as a means of destroying or controlling biofilms, the technology for this has not yet been successfully developed and relatively little

information is available on the action of bacteriophage on biofilms (Hughes *et al.,* 1998; Sutherland *et al.,* 2004).

2.7.3 Biofilm control by means of interspecies interactions – bioregulation

The existence of multiple interactions or the simple production of a metabolite can interfere with the development of what seems to be structurally organized communities existent within a biofilm. Competition for substrate is considered to be one of the major evolutionary driving forces in the bacterial world, and numerous experimental data obtained in the laboratory under well-controlled conditions show how different microorganisms may effectively out compete with others because of better utilization of a given energy source (Christensen *et al.,* 2002). Furthermore, many bacteria are capable of synthesizing and excreting surfactants. In a competitive environment this phenomenon could play a significant role. Al-Tahhan *et al.* (2000) pointed out that even very low levels of a rhamnolipid biosurfactant, synthesized by a *Pseudomonas* spp., could render the cell surface more hydrophobic. It has been suggested that biosurfactants might be involved in the transfer of exopolymer from one bacterial species to another, taking place more efficiently within the matrix of a biofilm where the cells are in close proximity to each other (Osterreicher-Ravid *et al.,* 2000). Nevertheless, in a mixed species biofilm, this cell feature promoted by a bacterial specie could have antimicrobial properties to the others species. The production of biosurfactants can impair the formation of biofilms (Daniels *et al.,* 2004). Several authors (Leriche and Carpentier, 2000; Zhao *et al.,* 2004) found that the microorganisms comprising the biofilms on dairy plant surfaces could play a role by interfering with the biological activities of pathogenic bacteria and therefore, could be used to improve the hygiene of surfaces. Surfactin from *Bacillus subtilis* disperses biofilms without affecting cell growth and prevents biofilm formation by microorganisms such as *Salmonella enterica*, *Escherichia coli*, and *Proteus mirabilis* (Mireles *et al.,* 2001). Also, lactic acid bacteria and their products have been well documented for their antimicrobial activity against the growth of *Listeria monocytogenes* (Zhao *et al.,* 2004).

The discovery that wide spectrums of bacteria use quorum sensing to perform biofilm formation and differentiation makes it an attractive target for biofilm control (Cui, 2003; Daniels *et al.,* 2004).

2.8 Biofilm control – cleaning and disinfection

2.8.1 Biocidal products - definition

According to the Directive 98/8/EC of the European Parliament and of the Council of 16 February 1998, concerning the placing of biocidal products on the market, biocides are active substances and preparations containing one or more active substances, put up in the form in which they are supplied to the user, intended to destroy, deter, render harmless, prevent the action of, or otherwise exert a controlling effect on any harmful organism by chemical or biological means.

The term **biocide** is commonly used as synonym of antimicrobial agent or disinfectant/sanitizers. Nevertheless, according to Gilbert and McBain (2003) the three terms are differentiated and defined as:

Biocides are active substances that above certain concentrations and defined conditions will kill cells within specified times;

Antimicrobial agents are active substances that have adverse effects on the growth or survival of microorganisms;

Disinfectants/sanitizers are formulations containing active substances that are safe for the application to inanimate surfaces and which kill specified groups of disease-producing microorganisms within specified times.

2.8.2 Environmental parameters required for biocides use

The above mentioned Directive proposes that a biocidal product showed obey to the following characteristics:

- is sufficiently effective;
- has no unacceptable effects on the target organisms, such as unacceptable resistance or cross-resistance;
- has no unacceptable effects itself or as a result of its residues, on human or animal health, directly or indirectly (*i.e.,* through drinking water, food or feed, indoor air or consequences in the place of work) or on surface water and groundwater;
- has no unacceptable effect itself, or as a result of its residues, on the environment having particular regard to the following considerations:

  - its fate and distribution in the environment; particularly contamination of surface waters (including estuarian and seawater), groundwater and drinking water;
  - its impact on non-target organisms;
- its physical and chemical properties have been determined and deemed acceptable for purposes of the appropriate use, storage and transport of the product.

2.8.3 Cleaning and disinfection

In food industry the operations of cleaning and disinfection are essential parts of food production and the efficiency with which these operations are performed greatly affects final product quality (Carpentier and Cerf, 1993). Cleaning and disinfection operations will be considered separately, even thought in practice they are sometimes hard to dissociate.

2.8.3.1 Cleaning

Cleaning is an important stage for minimizing microbial colonization of industrial food processing equipment (Carpentier and Cerf, 1993). It seems to be of fundamental importance to eliminate as many microorganisms as possible before applying a disinfectant. Cleaning procedures should effectively remove food debris and other soils that may contain microorganisms or promote microbial growth. Most cleaning regimes include removal of loose soil with cold or warm water followed by the application of chemical agents, rinsing, and disinfection. High temperatures can reduce the need for physical force (Carpentier and Cerf, 1993; Maukonen *et al.,* 2003). Chemical agents, usually surface active agents or alkali compounds, used as detergents, suspend and dissolve food residues by decreasing surface tension, emulsifying fats, and denaturing proteins (Maukonen *et al.,* 2003). These chemical agents are currently used in combination. Many situations, in the dairy industry, require the occasional use of acid cleaners to clean surfaces soiled with precipitated minerals or having high food residue/mineral content (such as milkstone). Mechanical action (water turbulence and scrubbing) are recognized as being highly effective in eliminating biofilm (Chmielewski and Frank, 2003; Maukonen *et al.,* 2003). An effective cleaning procedure must break up or

dissolve the EPS matrix associated with the biofilm so that disinfection agents can gain access to the viable cells (Carpentier and Cerf, 1993; Gibson *et al.,* 1999).

The cleaning process can remove 90 % or more of microorganisms associated with the surface, but cannot be relied upon to kill them. Bacteria can redeposit at other locations and, given time, water and nutrients can form a biofilm. Therefore, disinfection must be implemented (Gibson *et al.,* 1999). Other drawback of a cleaning process is that it is often impracticable and can be costly because it usually involves equipment downtime, being biocides and antibiotics the main weapons to control biofilms (Srinivasan *et al.,* 1995).

In dairy and food processing units, cleaning is very important and it is imperative that all food-contact surfaces of processing and handling equipment be clean and hygienic, to prevent microbial contamination and to produce quality food products. However, to achieve this goal, the cleaning system should include a specific sequence of cleaning agents and sanitizers applied by defined time–temperature combinations after the use of the equipment. In practice, much attention is given to the cleaning and sanitizing operations within the cleaning system. These are complementary processes which together help to achieve the desired results.

2.8.3.2 Disinfection

Disinfection is the use of products (disinfectants) to destroy microorganisms. Nevertheless, microorganisms have been found in disinfectant solutions, which is due to their ability to form resistant strains and build-up protective biofilms (Gilbert and Allison, 1999; McBain *et al.,* 2000). This means that microbial contamination can be spread on the surface to be disinfected instead of being disinfected.

In food plant operations, disinfection is required, since wet surfaces provide favourable conditions for the growth of microorganisms (Maukonen *et al.,* 2003). The aim of disinfection is to reduce the surface population of viable cells after cleaning and prevent microbial growth on surfaces before restart of production. Disinfectants do not penetrate the biofilm matrix left on a surface after an ineffective cleaning procedure, and thus do not destroy all the living cells in biofilms (Holah, 1992; Carpentier and Cerf, 1993). Disinfectants are more effective in the absence of organic material (fat, carbohydrates, and protein based materials). Interfering organic substances, pH, temperature, water hardness, chemical inhibitors, concentration and contact time generally control the efficacy of

disinfectants (Mosteller and Bishop, 1994; Cloete *et al.,* 1998). The disinfectants must be effective, safe and easy to use, and easily rinsed off from surfaces, leaving no toxic residues that affect the sensory values of the product. The use of disinfectants in food plants depends on the material used and the adhering microorganisms. The disinfectants to be used should be chosen based on the following statements (Troller, 1993; Wirtanen, 1995; Wirtanen *et al.,* 2000):

- is the disinfectant effective in the pH range used?
- is the disinfectant stable when diluted?
- is the disinfectant toxic, safe or irritating?
- what is the microbial spectrum of the disinfectant?
- how does the temperature affect the activity of the disinfectant?
- is the disinfectant corrosive at the surface?
- is the disinfectant surface active?
- is the disinfectant stable when reacting with organic material?

Table 2.5 resumes the properties of disinfectants commonly used in industrial systems.

Table 2.5 Properties and uses of chemical disinfectants (based on Troller, 1993; Banner, 1995; Wirtanen, 1995)

Disinfectant type	Applications	Limitations	Comments
Chlorine	Neutral/alkaline conditions; Stainless steel; Food contact surfaces; Floors/walls/air; CIP, spray, soak, fog	Acid conditions; High temperature; Soft metal; mild steel; Vapour phase corrosion	Broad spectrum kill; Organic Cl less corrosive; Taste/odour carryover; Irritates eyes, skin, etc; Environmentally unfriendly; Cheap
Chlorine dioxide	Water treatment/slime/odour control; Rinse for fruit/vegetables; Acid form on food contact surfaces; Stainless steel; CIP, spray, soak	Stabilized form slower and less active under alkaline/neutral conditions; More active acid form requires activating acid, mixing step; More expensive than chlorine; Hazards of generating gas; Irritant to workers	Acidified form is broad spectrum; Does not form chloramines or trihalomethanes; Uncertain corrosion properties

Iodine	Acid conditions, < pH 3; Stainless steel, plastics; Food contact surfaces; Floors/walls; CIP, spray, soak, manual; Hand disinfectant; Carbon dioxide atmosphere; Helps dissolve mineral deposits	Less effective than chlorine; Activity declines rapidly above pH 5; Corrosive to soft metals and mild steel; Stains certain plastics; Potential taste/odour carryover; Most foam upon circulation	Broad spectrum kill; Organic soil reduces efficacy; More costly than chlorine but effective at lower concentration
Anionic surfactants at acid conditions	Acid conditions, < pH 3; Carbon dioxide atmosphere; Stainless steel, plastics; Foam on external surfaces; CIP, spray, soak, manual; Carbon dioxide atmosphere; Overnight disinfection; Milkstone/beerstone removal	Effective only at low pH; Neutralized by detergent residues; Corrosive to soft metals, mild steel; Some products moderate to high form upon circulation	Moderate to high foam production; Stable at high temperature; No odour/taste carryover; More costly than halogens
Peracetic acid	Acid conditions; Carbon dioxide atmosphere; Stainless and mild steel, soft metals, plastic, rubber; Food contact surfaces; CIP, spray, soak	Corrosive in presence of chlorine ions; Concentrate causes burns, irritations; Pungent odour; Rapid decomposition at high temperatures, generates gas and heat; Rapid decomposition by metals, organic matter	Broad spectrum kill; Safe decomposition products; Concentrate should not contact mild steel, soft metals; No phosphates; Moderate cost
Quaternary ammonium compounds (cationic surfactants)	Neutral/alkaline conditions; Applicable to all materials; Food contact surfaces; Environmental areas/residue can extend activity; Mildew and odour control; Water treatment; Spray, soak, manual, circulation	Selective germicidal activity; Residual fil affects cheese starter cultures; Moderate to high foam upon circulation; Toxicity at high concentrations; Neutralized by certain surfactants	Properties vary among different QAC´s; Effective for wetting and penetrating soils; Effective in presence of organic soils; Non-irritating; Newer QAC´s have high water hardness tolerance
Amphoteric surfactants	Neutral/alkaline conditions; Applicable to all materials; Food contact surfaces; Environmental areas; Spray, manual soak; Fog air;	Reduced activity under acid conditions; Selective germicidal activity; Residual film affects cheese starter cultures; Used at high concentrations;	No odour/taste carryover; Low toxicity; Non-irritating

	Foam is suitable for external surface disinfection	Moderate to high cost; Moderate to high foam	
Polymeric biguanides	Acid/alkaline conditions; Applicable to all materials; Food contact surfaces; Environmental areas; Can/bottle warmers, water treatment; Spray, soak, manual, circulation; Fog air	Limited activity; Incompatible with anionic surfactants; Moderate to high cost	Activity reduced by organic soil
Glutaraldehyde	Neutral/alkaline conditions; Non-corrosive to all materials; Water treatment/slime control in can/bottle warmers, tunnel pasteurizers; Glycol and sweetwater systems in dairies; Conveyor lines	Slower, less active under acid conditions; Absorbed by porous materials	Broad spectrum kill; Less toxic and irritating and less offensive odour than formaldehyde; Toxicity at high concentrations
Isothiazolinones	Acid, alkaline, neutral conditions; Applicable to all materials; Cooling water/towers, can/bottle warmers; Long-term, continuous activity; Conveyor lubricants	Slowly active; Use on non-food contact surfaces	Broad spectrum kill; More active under acid conditions
Phenolics	Lubricants for conveyor lines; Water treatment	Variable germicidal activity; Toxicity; Irritates body tissue; Readily absorbed by many materials; Unpleasant odours	
Hydrogen peroxide	Applicable to all materials; Sporicide at high concentration at high temperature; Aseptic packing of beverages	Weak germicidal properties; Efficacy at high temperatures/high concentration; Destabilized by metal contaminants (copper, iron)	Safe decomposition products, water and oxygen

2.9 Mode of action of antimicrobial agents

The processes involved in the antimicrobial action comprise transportation of the antimicrobial agent to the surface of the cell, adsorption, diffusion, penetration and interaction at the target site. These processes are not instantaneous, the time they take, and the correspondent killing time may differ within antimicrobial agents. The differences also depend on the mode of action, as well as on the chemical constitution and chemico-physical properties of the chemical (Paulus, 1993).

An antimicrobial effect can be defined as an interaction between an active substance and specific targets of the microbial cell. Critical governing features of these interactions are the physicochemical characteristics of the chemical agent, cell morphology, and the physiology status of the microorganism (Paulus, 1993; Denyer and Setwart, 1998). In Figure 2.6 are depicted the targets of action of several antimicrobial agents against microorganisms.

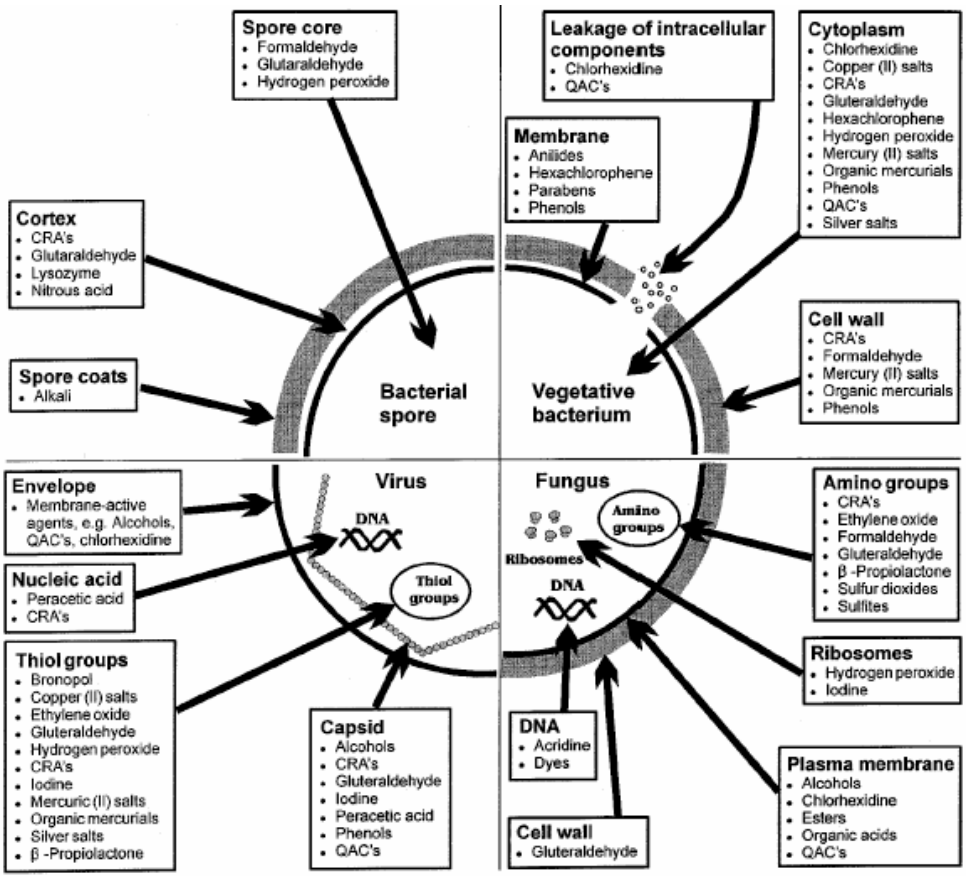


Figure 2.6 Mechanisms of microorganism inactivation by antimicrobial agents (according to Cloete *et al.,* 1998; Cloete, 2003). CRA´s = chlorine-releasing agents; QAC´s = quaternary ammonium compounds.

Antimicrobial agents derive from a variety of chemical classes. The precise mechanisms of interaction often reflect this diversity, although the final damage outcomes may show considerable similarity. Taking into account Figure 2.6 and according to Denyer and Stewart (1998), antibacterial events include:

- Disruption of the transmembrane proton motive force leading to an uncoupling of oxidative phosphorylation and inhibition of active transport across the membrane;
- Inhibition of respiration or catabolic/anabolic reactions;
- Disruption of replication;
- Loss of membrane integrity resulting in leakage of essential intracellular constituents such as potassium cation, inorganic phosphate, pentoses, nucleotides and nucleosides, and proteins;
- Lysis;
- Coagulation of intracellular material.

2.10 Factors influencing the efficacy of antimicrobial agents - environmental conditions

The efficacy of the application of a disinfection procedure is the result of the correct application of an efficient antimicrobial agent. That means that lack of efficacy may have multicasual explanations and resistance is only one of them. There are some reasons to cause loss of efficacy in a disinfection process which may be misunderstood as resistance. They can be summarized as follows (Bessems, 1998; Heinzel, 1998; Russell, 2003):

- Use of an inefficient product, *i.e.,* an antimicrobial agent which has an incomplete spectrum of activity. By this way all microorganisms which are outside the range of product efficiency will survive;
- Application of the product without regard to the correct conditions as recommended by the supplier. This concerns mainly concentration, pH, temperature, and time of application but also inactivation by organic matter or other products;
- The prolonged application of antimicrobial agents at sublethal concentrations may provoke the adaptation of microorganisms to these antimicrobial agent and possibly to other antimicrobial agents;
- Insufficient contact of the antimicrobial agent with the microorganisms;

- Insufficient availability of the antimicrobial agent.

All these circumstances may diminish the expected action of the antimicrobial agents.

Nevertheless, the main reasons for the failure of a disinfection procedure are linked with the development of microbial resistance to antimicrobial agents. According to Gilbert and McBain (2003), resistance is a description of the relative insusceptibility of a microorganism to a particular treatment under a particular set of conditions. For antimicrobial agents, it is usually quantified as the concentration that caused sublethal effects on the population cells. Besides the environmental factors, resistance to antimicrobial agents can be a natural property of a microorganism (intrinsic) or acquired by mutation or acquisition of plasmids - self-replication, extrachromosomal DNA or transposons - chromosomal or plasmid integrating, transmissible DNA cassettes (McDonnell and Russell, 1999; White and McDermott, 2001; Gilbert *et al.,* 2002; Cloete, 2003; Gilbert and McBain, 2003).

2.11 Mechanisms of cellular resistance to antimicrobial agents

2.11.1 Reduced susceptibility to antimicrobial agents associated with genotypic changes (acquired mechanisms)

Reduced susceptibility of microorganisms to antimicrobial agents may be acquired through mutation, or by the acquisition of a plasmid or transposon (Beumer *et al.,* 2000; White and McDermott, 2001; Gilbert and McBain, 2003). Chromosomal gene mutations conferring resistance to antibiotics are relatively well studied (McDonnell and Russell, 1999; Beumer *et al.,* 2000; White and McDermott, 2001). By contrast, fewer studies have been performed to determine whether mutation confers resistance to biocides or to disinfectants (McDonnell and Russell, 1999). The mutation that alters the target site of an antimicrobial agent, which acts at specific sites within the bacterial cell, is likely to cause resistance. These mechanisms of resistance are observed through the existence of bypass in the bacterial ribosome or a metabolic enzyme, or overproduction of the target enzyme or of an efflux pump (Beumer *et al.,* 2000).

Acquired resistance arises *via* mutation or as result of the acquisition of genetic elements - plasmids, transposons (White and McDermott, 2001). Acquired, nonplasmid-

encoded resistance may result when bacteria are trained to grow in gradually increasing concentrations of a biocide, although, resistance is not always stable. Temporary resistance by phenotypic adaptation is known, but is considered that, in general, a nongenetic adaptative type of resistance is unlikely to play an important role in determining the long-term survival of bacteria to antimicrobial agents (Russell, 2001).

2.11.2 Intrinsic properties of microorganisms conferring reduced susceptibility to antimicrobial agents

Intrinsic insusceptibility is a natural property of a microorganism and is shown by bacterial spores, mycobacteria, and several Gram-negative bacteria. As summarized in Figure 2.7 Gram-negative bacteria are generally relatively less susceptible to antimicrobial agents than Gram-positive bacteria because their cell walls present a more significant barrier to entry (McDonnell and Russell, 1999). The outer membrane of Gram-negative bacteria acts as a permeable barrier because the narrow porin channels limit the penetration of hydrophobic molecules and the low fluidity of the lipopolysaccharides leaflet slows down the inward diffusion of lipophilic compounds (McDonnell and Russell, 1999; Cloete, 2003). Mycobateria, which possesses a waxy envelope that inhibits the uptake of some antimicrobial agents, are even more resistant (McDonnell and Russell, 1999). The coat and the cortex of bacterial spores present a barrier to the entry of antimicrobial agents, explaining their relatively extreme insusceptibility (McDonnell and Russell, 1999; Cloete, 2003).

When spores germinate, the biochemical and structural changes that follow often results in the germinating cells becoming more susceptible to the action of some chemical compounds (Beumer *et al.,* 2000). So, the intrinsic microbial resistance is frequently associated with cellular impermeability imparted by the outer layers of a bacterial cell that limit the uptake of antimicrobial agents, although active efflux pumps appears to be an important process (Russell, 2001; Cloete, 2003; Gilbert and McBain, 2003). Efflux is increasingly implicated as a resistance mechanism. Efflux pumps contribute to the intrinsic resistance of Gram-negative bacteria by pumping out a variety of agents, including dyes, detergents and antibiotics (Beumer *et al.,* 2000; Cloete, 2003). Efflux pumps are recognised as common membrane components in all cell types, from prokaryotes to superior eukaryotes (van Bambeke *et al.,* 2003). It confers bacteria a common and basic mechanism of

resistance by extruding toxic molecules (Cui, 2004). In the context of antibiotic resistance, the term multidrug resistance (Mdr) is used to describe a situation where reduced susceptibility to an antibiotic is associated with reduced susceptibility to other chemically unrelated antibiotics through an efflux mechanism. There is evidence that *P. aeruginosa* can efflux triclosan and that this represents an important intrinsic susceptibility to bisphenol (Chuanchuen *et al.,* 2001). The presence of the efflux systems coupled with the narrow porin channels in the outer membrane of the cell which restricts diffusion of antimicrobial agents into the cells is responsible for the very high intrinsic resistance of Gram-negative bacteria to antimicrobial agents (Beumer *et al.,* 2000; Cloete, 2003).

As well as impaired uptake or increased efflux, some microorganisms demonstrate intrinsic resistance through the inactivation of antimicrobial agents (Beumer *et al.,* 2000). Some bacteria can degrade antimicrobial agents (Nishihara *et al.,* 2000; Gilbert and McBain, 2003) and this phenomenon does not appear to be any plasmid involvement, representing another example of intrinsic insusceptibility.

Physiological (phenotypic adaptation of microorganisms that reduces susceptibility to antimicrobial agents in response to environmental changes is also considered as intrinsic resistance (McDonnell and Russell, 1999). The cell phenotype expressed can vary significantly depending on the environmental conditions under which it is grown (White and McDermott, 2001; Russell, 2003). The resistance of these microorganisms to antimicrobial agents may derive partly from changes in outer cell layers that increase the barrier properties and prevent access to their site of action, but other changes are also involved (McDonnell and Russell, 1999; White and McDermott, 2001; Cloete, 2003; Russell, 2003). The association of microorganisms with solid surfaces leads to the formation of a biofilm, with bacteria in different zones of the biofilm experiencing different nutrient environments and displaying different physiological properties (Gilbert *et al,* 1990; Brown and Gilbert, 1993; McDonnell and Russell, 1999). Reduced susceptibility of bacteria in biofilms to antimicrobials can sometimes be extreme and is probably caused by a variety of factors including nutrient depletion within the biofilm, reduced access of the antimicrobial agent to cells in the biofilm, chemical interaction between the chemical agent and the biofilm, and the production of degradative enzymes and neutralizing chemicals (Brown and Gilbert, 1993). Biofilms have been reported as possessing susceptibilities towards biocides and antibiotics that are 100 – 1000 times less than equivalent populations of planktonic bacteria (Gilbert *et al.,* 2002).

The phenomenon involved in the resistance of biofilms to antimicrobials agents will be consistently developed ahead on this chapter.

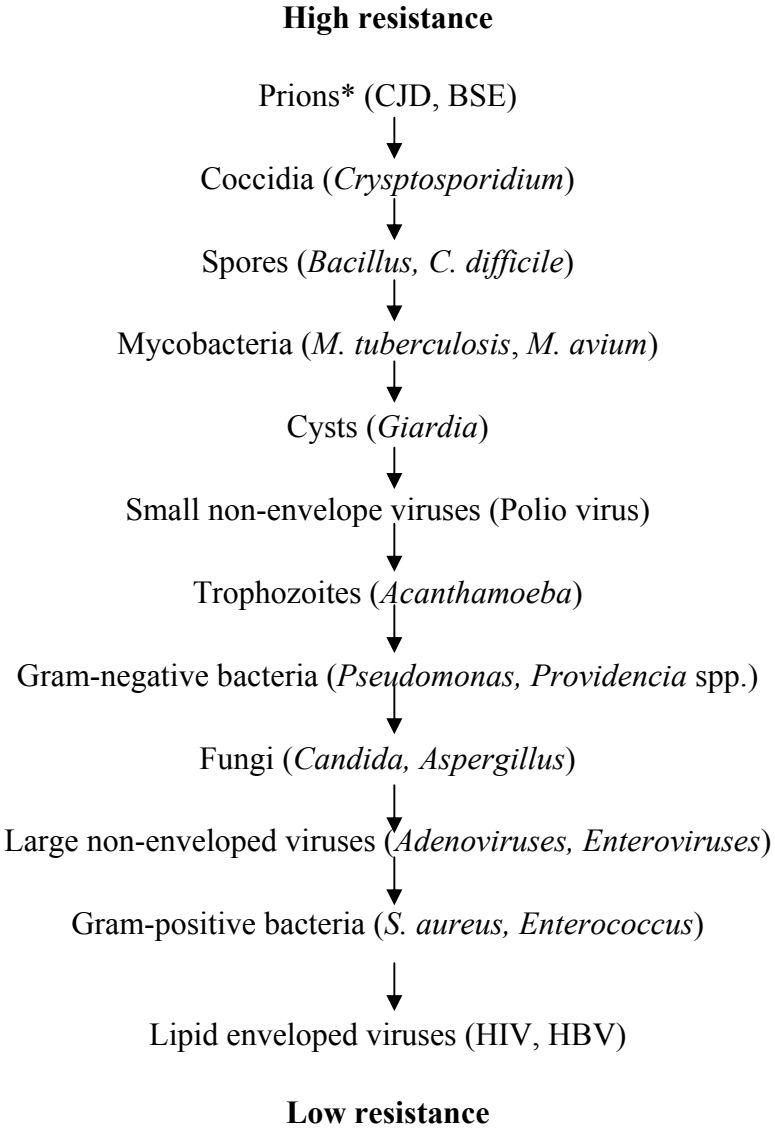


Figure 2.7 Descending order of resistance to disinfectants. The asterisk indicates that the conclusions are not yet universally agreed upon (based on McDonnell and Russell, 1999).

2.12 Mechanisms of biofilm resistance to antimicrobial agents

There is no one answer to the question of why and how bacteria growing in a biofilm develop increased resistance to antimicrobial agents. In the following statements will be described some of the possible mechanisms that can account for the resistance of bacteria within biofilms to antimicrobial compounds.

2.12.1 Resistance and the extracellular polymeric matrix

It has been suggested and previously described in sub-chapter 2.3.2 that the extracellular polymeric matrix, among other functions, prevents the access of antimicrobial agents to the cells embedded in the biofilm community (Mah and O´Toole, 2001). The presence of a charged, hydrated exopolymer matrix around individual cells and microcolonies profoundly affects the access of antimicrobial agents. Restricted diffusion from the surrounding medium, by a combination of ionic interaction and molecular sieving events, may occur for appropriate classes of molecules (Costerton *et al.,* 1987). The constituents of the biofilm matrix act as would an ion exchange resin and actively remove strongly charged molecules (Gilbert *et al.,* 2002). Total penetration failure will only occur when the reaction sites are sufficient to deplete the bulk concentration of the antimicrobial agent or replenishment of the matrix proceeds at a faster rate than does adsorption/reaction and diffusion (Stewart *et al.,* 1998; Gilbert *et al.,* 2002). Diffusion limitation studies have generally focused on antibiotics rather than biocides and upon medically relevant biofilm populations rather than biofilms in industrial situations (Stewart, 1996).

In addition to the potential of the biofilm matrix to react directly and chemically quench reactive moieties, retention of enzymes with the capability to inactivate antimicrobial agents within the biofilm matrix will amplify its barrier properties with respect to the diffusion of suitable substrates (Heinzel, 1998; Gilbert *et al.,* 2002).

2.12.2 Resistance associated with growth rate and nutrient availability

When a bacterial cell culture becomes starved for a particular nutrient, it slows its growth. Transition from exponential to slow or no growth is generally accompanied by an increase in resistance to antimicrobial agents (Wentland *et al.,* 1996; Lewis, 2001). Because

cells growing in biofilms are expected to experience some form of nutrient limitation, it has been suggested that this physiological change can account for the resistance of biofilms to antimicrobial agents (Mah and O`Toole, 2001). Desai *et al.* (1998) compared the resistance of planktonic and biofilm cells at different stages during exponential growth up to the entry into stationary phase. They found that resistance increased as the planktonic cultures and the biofilm cells approached stationary phase. The maximal resistance of both cultures occurred in stationary phase where the biofilm cells were about 15 times more resistant than the planktonic cells. These results suggested that some determinant other than growth rate is responsible for a certain level of resistance and slow growth adds additional protection (Fux *et al.,* 2005a).

Oxygen gradients within the biofilm may also directly influence the activity of some antibacterial agents (Gilbert *et al.,* 2002). Another phenomenon associated with biofilm is the existence of physiological gradients across biofilms on growth and metabolism of cells at the periphery to consume nutrients before they permeate to the more deeply placed cells. The peripheral cells will have growth rates and nutrient profiles that are similar to those of planktonic cells, allowing for the existence of heterogeneity within biofilm. Advances in technology have resulted in the ability to visualize the heterogeneity within a biofilm (Fux *et al.,* 2005a). A staining method utilizing acridine orange was employed to identify regions of biofilms that contained rapidly or slowly growing cells based on their relative RNA-DNA content (Wentland *et al.,* 1996). The environmental heterogeneity that exists within a biofilm might promote the formation of a heterogeneous population of cells, such different levels of resistance can be expressed throughout the community (Wentland *et al.,* 1996; Fux *et al.,* 2005a). So, a major contributor towards the inefficacy of antimicrobial treatments when applied to biofilms must, therefore, be associated with physiological heterogeneity (Allison *et al.,* 2000; Mah and O`Toole, 2001).

2.12.3 Resistance associated with the adoption of resistance phenotypes

Bacteria can sense the proximity of a surface, up-regulate production of EPS and rapidly alter their susceptibility to antimicrobial agents after binding. In some instances, 3 to 5 fold decreases in susceptibility occurred immediately on attachment in the presence of antimicrobial agents that exceeded the minimum inhibitory concentration for planktonic cells (Gilbert *et al.,* 2002; Fux *et al.,* 2005a). The magnitude of the decreases in

susceptibility observed immediately after bacterial attachment, but before biofilm formation, is generally far less than that observed in mature biofilms and is insufficient to account for the reported levels of resistance in biofilm communities (Gilbert *et al.,* 2002).

The microorganisms generate physiological changes that act to protect the cell from various environmental stresses. Thus, the cells are protected from the detrimental effects of heat shock, cold shock, changes in pH and many chemical agents. Nevertheless, the physiological changes begin when cells attach to a surface, by expressing a biofilm phenotype that can confer resistance face to stress environmental conditions (Mah and O`Toole, 2001; Gilbert *et al.,* 2002). This resistant phenotype might be induced by nutrient limitation, certain types of stress, high cell density, efflux of the treatment agent or a combination of these phenomena (Mah and O`Toole, 2001).

The role of quorum sensing in antimicrobial resistance is not yet clear. It has been suggested that regulation of EPS, under the control of signal molecules such as *N*-acyl homoserine lactones is responsible for the early transcriptional events associated with biofilm formation (Davies *et al.,* 1998). Such global regulators are responsive to increases in cell density, beyond critical threshold values, and may be general regulators of biofilm-specific physiology (Davies *et al.,* 1998). In biofilms, signal molecules would become concentrated within geometric centre of biofilm, thereby increasing EPS production. This would alter the distribution and density of cells throughout the matrix and confer some level of structural organization upon the community to provide customized microniches at various points within the biofilm (Gilbert *et al.,* 2002). Brooun *et al.* (2000) showed that *P. aeruginosa* mutants defective in quorum sensing were unaffected in their resistance to detergents and antibiotics. Nevertheless, Mah and O`Toole (2001) suggest that additional experiments are required to elucidate the role of quorum sensing in antimicrobial resistance.

Sublethal concentrations of antimicrobial agents might act as inducers/transcriptional activators of more tolerant phenotypes, such as those expressing the multidrug resistance operons and efflux pumps in *E. coli* (Ma *et al.,* 1993; Maira-Litran *et al.,* 2000).

A novel hypothesis for the considerable recalcitrance of biofilm relates to the potential of damaged bacterial cells to undergo apoptosis or programmed cell death. Lewis (2000; 2001) suggested that death of cells following treatment with antimicrobial agents results not from direct action of the agent but from a programmed suicide mechanism and cellular lysis. Following the absence of an adverse condition, the damaged persistent cells would grow rapidly in the presence of nutrients released from their lysed community

partners and the community would become restored. These cells would survive treatment phases and proliferate in the post-treatment phase, thereby stimulating considerable recalcitrance upon the biofilm community.

Chapter 3

Material and methods

Abstract

This chapter describes the general methodology used to perform the work presented in the several chapters of results that constitute this thesis.

3.1 Microorganism and culture conditions

Three different *Pseudomonas fluorescens* strains were used throughout this work:

The type strain purchased from the American Type Culture Collection, *Pseudomonas fluorescens* ATCC 13525^T;

Pseudomonas fluorescens D3-348 and *Pseudomonas fluorescens* D3-350, two strains isolated from an overhead pipe filler in a dairy processing plant (Dogan and Boor, 2003), gently provided by Kathryn J. Boor from the Department of Food Science, Cornell University, Ithaca, New York.

The isolated strains were selected due to their similar source (overhead pipe filler – drain samples) and significant differences in their ribotype and extracellular-enzyme production (Dogan and Boor, 2003). *P. fluorescens* D3-348 (ribotype – 536-S-8) was protease and lipase negative, and lecithinase positive, while *P. fluorescens* D3-350 (ribotype – 112-S-2) was positive for protease, lecithinase and lipase.

3.1.1 Bacteria preservation

All the bacteria were criopreserved in criovials (Nalgene) in a refrigerate chamber at -80 ± 2 ºC.

Bacteria reactivation was made by striking 10 µl of the bacterial suspension existent in the criovial to solid media consisting of 0.5 % (w/v) glucose (Merck), 0.25 % (w/v) peptone (Merck), 0.125 % (w/v) yeast extract (Merck) and 1 % (w/v) agar (Merck) and incubated in a stove (Memmert, model B 40) at 27 ºC during 24 h.

3.1.2 Culture medium

P. fluorescens were allowed to grow in a sterile (autoclaved at 121 ºC for 20 min) synthetic aqueous nutrient medium consisting in 0.5 % (w/v) glucose, 0.25 % (w/v) peptone, 0.125 % (w/v) yeast extract in phosphate buffer (0.2 M KH_2PO_4; 0.2 M Na_2HPO_4 - Merck) pH 7 ± 0.2.

3.2 Experimental conditions for biofilm formation

3.2.1 Inoculum preparation

A bacterial suspension was prepared by gently removing bacteria from the solid media using a sterile metal device, and immersing this device into a 500 ml flask containing 200 ml of sterile nutrient medium. This bacterial suspension was incubated at 27 ºC with agitation (B. Braun Biotech International, model CERTOMAT® M and CERTOMAT® S) at 120 min^{-1} during 12 h, in order to have bacteria in the exponential phase of growth. Afterwards, the inoculum was transferred to a reactor in a volume of 10 % of the useful volume of the reactor.

3.2.2 Adhesion surface

Stainless steel was used throughout this work since this material is representative of the major part of the surfaces found in equipments in industrial systems (Pereira, 2001). The tests with biofilms were performed with flat stainless steel plates (ASI 316) with 1.25 cm × 1.75 cm and with round surfaces (cylinders of diameter = 2.2 cm and length = 5 cm). Before use, both types of surfaces were polished with alumina polishing suspension at 0.5 μm (Struers Cat. No. 40700037), being, thereafter, defatted using a commercial detergent, washed in order to remove the detergent and reserved in ethanol at 70 % (w/v) until use.

3.2.3 Continuous reactor

The bacterial cultures were maintained in a 2 l reactor (Pobel), operating continuously in order to provide *P. fluorescens* in the exponential phase of growth. This reactor was continuously aerated, agitated and maintained at 27 ºC. The aeration was provided with an aquarium air pump (Anivite, model Tagus 2000), being the air previously filtrated with a 0.2 μm cellulose acetate membrane (Merck). The agitation was carried out by putting the reactor, with a magnetic stirrer inside, on a plate with magnetic agitation and regulated velocity (Selecta, model Agimatic-S). Two drops of silicone antifoaming (Merck 7743) were placed in the reactor.

3.2.4 Reactor sterilization and operation

The reactor, containing the culture medium (sub-chapter 3.1.2) for bacteria growth, the magnetic stirrer, the system for air dispersion and the silicone tubes for nutrient and air supply, was sterilized in an autoclave at 121 ºC (AJC, model Uniclave 88) for 20 min.

The conditions of growth (aeration, agitation and temperature) were fixed and the reactor containing the sterile medium was inoculated with bacteria obtained according to sub-chapter 3.2.1.

Initially, the reactor operated in batch conditions during 12 h in order to have a high cellular concentration and the bacteria in the exponential phase of growth. Afterwards, the reactor operated continuously by feeding growth medium at a flow rate of 40 ml/h using a peristaltic pump (Ismatec, model Reglo Analog MS-2/6). The reactor was allowed to operate during one week, afterwards the system was sterilized at 121 ºC for 20 min.

The continuous operation of this reactor permitted to have a constant source of *P. fluorescens* cells in the exponential phase of growth, to use in the several experimental tests with bacteria in suspension.

3.3 Biofilm set-up – flow cell reactor

3.3.1 Association of the continuous reactor with a dilution reactor

A 0.5 l reactor operating continuously, and in the same conditions as the ones described above for the 2 l reactor (sub-chapter 3.2.3), was used for bacterial growth. In order to carry out the adequate dilution rate to promote biofilm formation, the bacterial suspension was diluted using a 3.5 l Perspex^{TM} reactor. This 3.5 l reactor was continuously inoculated with bacteria coming from the 0.5 l reactor (10 ml/h) using a peristaltic pump and feed with a minimal nutrient medium (1.7 l/h), consisting of 0.005 % (w/v) glucose, 0.0025 % (w/v) peptone, 0.00125 % (w/v) yeast extract in phosphate buffer (0.2 M KH_2PO_4; 0.2 M Na_2HPO_4) pH 7 ± 0.2. In these conditions, according to Pereira *et al.* (2002a), it was established an adequate bacterial concentration (6×10^7 cells/ml) and dilution rate suitable to promote biofilm formation. The bacterial suspension from this Perspex^{TM} reactor was used to feed the biofilm reactors (Figures 3.2, 3.3 and 3.4).

3.3.2 Flow cell reactor and biofilm formation

The diluted bacterial suspension, described above (sub-chapter 3.3.1), was pumped up, passing through the flow cell reactors and back to the Perspex™ reactor (Figure 3.2).

A flow cell reactor, described by Pereira *et al.,* (2002a), was used as the device for biofilm formation. It consists of a semicircular Perspex duct (45 cm length and 1.6 cm of hydraulic diameter) with 10 apertures on its flat wall, to suitably fit several removable rectangular pieces of Perspex™. These pieces of Perspex™ have glued in one of its faces stainless steel (ASI 316) slides - 1.75 cm × 1.25 cm (Figure 3.1) for bacteria adhesion.

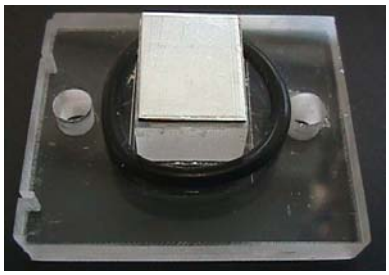


Figure 3.1 Photograph of a piece of Perspex™ with a stainless steel (ASI 316) slide glued.

Biofilms were formed on those metal slides whose upper faces were in contact with the bacterial suspension circulating in the flow cell reactor system (Figure 3.2). It was possible to remove separately each of the rectangular pieces without disturbing the biofilm formed on the others and without stopping the flow. This was managed because outlet ports were disposed on the round face of the flow cell between each two adjacent removable pieces of Perspex™ that allowed the deviation of the circulating flow from the point where the reactor was opened. The sampling was made from the top to the bottom and the rectangular piece removed was substituted with a new one that was previously cleaned and kept in ethanol.

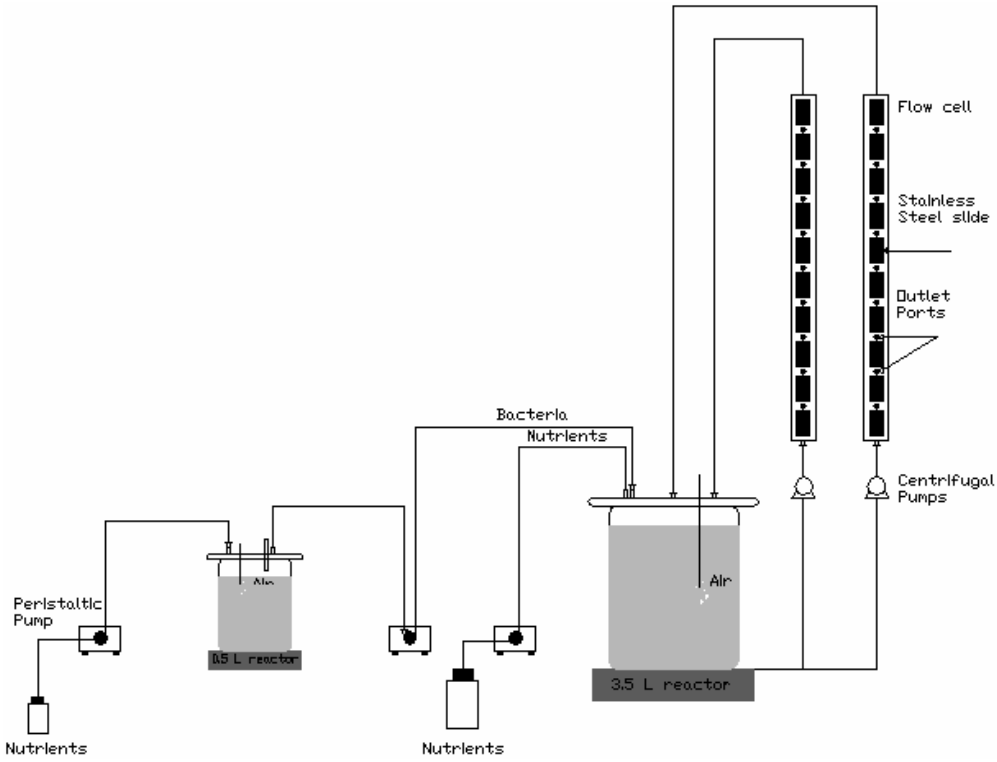


Figure 3.2 Schematic representation of the experimental apparatus system used to perform biofilm formation on the flow cell reactors.

Two parallel similar flow cell reactors (Figure 3.3) were used simultaneously in such a way that biofilms were formed under turbulent (Re=5200, u=0.532 m/s) and laminar (Re=2000, u=0.204 m/s) conditions, respectively, in each flow cell, that will be currently referred as turbulent and laminar biofilms. The bacterial suspension circulation was obtained using aquarium pumps (Eheim Typ 1060 and Typ 1048), being the pumps connected to the flow cells by means of silicone tubes (Figure 3.3). The biofilms were allowed to grow for 7 d to ensure that steady-state biofilms were used in every experiment (Pereira *et al*., 2002a).

Figure 3.3 Photograph of the flow cell reactor system.

3.3.3 Flow cell and dilution reactor disinfection

Since Perspex™ do not support high temperatures, the disinfection of the flow cell reactor was made by recirculation, in closed system, of a solution of 15 % (v/v) of sodium hypochlorite (10 ml/h) during 24 h. After the exposure to sodium hypochlorite, the system was washed, in open system, with sterile distilled water in order to remove the residual sodium hypochlorite. After the disinfection procedure, the tubes coming from the flow cells were aseptically placed in the 3.5 l Perspex™ reactor, being the system available to perform biofilm formation. The system was always subjected to a disinfection procedure before every new experiment.

3.4 Biofilm set-up – biofilm formation on a bioreactor rotating system

Biofilms were grown on stainless steel (ASI 316) cylinders, with a surface area of 34.6 cm^2 (diameter = 2.2 cm; length = 5 cm), inserted in a 3.5 l reactor, and rotating at 300 min^{-1} (Figure 3.4). Three stainless steel cylinders (Figure 3.5) were used in every

experiment. This reactor operated under the same conditions as the 3.5 l reactor associated with the flow cell reactors, *i.e.,* was continuously fed (1.7 l/h) with sterile diluted medium, using a peristaltic pump. It was also continuously inoculated with *P. fluorescens*, in the exponential phase of growth, supplied by the above referred 0.5 l reactor (sub-chapter 3.3.1), at a flow rate of 10 ml/h. The biofilms were allowed to grow for 7 d in order to obtain steady-state biofilms (Pereira *et al.,* 2001a).

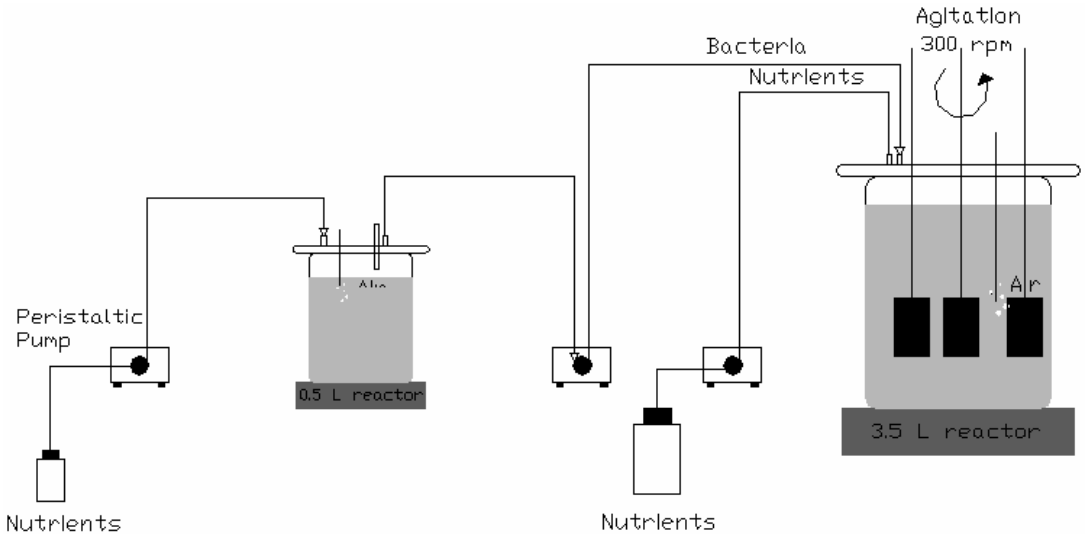


Figure 3.4 Schematic representation of the experimental system used to perform biofilm formation on the bioreactor rotating device.

Figure 3.5 Photograph of a stainless steel (ASI 316) cylinder.

3.4.1 Mechanical stability of the biofilm

The mechanical stability of the biofilms was assessed by means of determining the biomass loss due to the exposure of biofilms to increasing rotating speeds in the rotating device (Azeredo and Oliveira, 2000). Experiments were carried out with biofilms challenged by several chemical compounds, in independent assays.

3.5 Antimicrobial agents tested

In the present work were used two non-oxidizing biocides, two surface active agents and two oxidizing biocides. A brief description of these chemicals is presented bellow.

Non-oxidizing aldehyde-based biocides:

Glutaraldehyde (GTA) that was purchased from Reidel-de-Haën (Cat. No. 62621).

Ortho-phthalaldehyde (OPA) that was purchased from Sigma (Cat. No. P-1378).

Aldehydes belong to the group of electrophilic active agents which, due to the electron deficiency at the carbonyl carbon atom can react with nucleophilic cell entities and thus exert antimicrobial activity. Examples of nucleophilic reaction partners in the cell are amino and thiol groups, as well as the amide groups of amino acids or proteins. The latter in turn, are components of enzymes, which are inactivated by the reaction of their nucleophilic groups with aldehydes (Hugo and Russell, 1982).

GTA and OPA are dialdehyde compounds, which have two toxophoric groups that are responsible for the antimicrobial effect, since they can react with nucleophilic centres of the microorganism. Figures 3.6 and 3.7 present, respectively the molecular structure of GTA and OPA.

Figure 3.6 Molecular structure of glutaraldehyde.

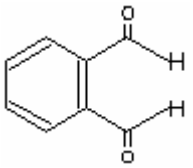


Figure 3.7 Molecular structure of *ortho*-phthalaldehyde.

Aliphatic aldehydes with molecular weights higher than the adipic aldehyde display no noticeable antimicrobial effect (Paulus, 1993). Conversely, aromatic dialdehydes exhibit antimicrobial properties. These aldehydes have been gained, in practice, utility as disinfectants (Walsh *et al.,* 1999a; 1999b; Simons *et al.,* 2001).

Surface active agents:

Sodium dodecyl sulfate (SDS), an anionic surfactant, purchased from Riedel-de-Haën - critical micellar concentration (CMC) - 8.30 mM; Cat. No. 62862.

Cetyltrimethyl ammonium bromide (CTAB), a cationic surfactant, purchased from Merck - CMC - 1.00 mM; Cat. No. 102342.

Surfactant is an abbreviation for surface active agent, which literally means active at a surface and is characterized by its tendency to absorb at surfaces and interfaces (Jönsson *et al.,* 1998). So, they are characterized by their ability to reduce the surface tension of aqueous fluids. Surfactants are constituted by two molecules with two different structural elements: a hydrophobic hydrocarbon (water repellent) group; and a hydrophilic polar (water attracting) group. Depending on the charge of the hydrophilic structural element, surfactants are classified as anionic, cationic, non-ionic and amphoteric or zwetterionic (Paulus, 1993; Rossmoore, 1995; Jönsson *et al.,* 1998).

Anionic surfactants, as SDS (Figure 3.8), exhibit some antimicrobial effect only in acid media (pH 2-3) that means in their undissociated state, but they have strong detergent properties (Hugo and Russell, 1982; Rossmoore, 1995). They present themselves as alkali or amine salts of long-chain fatty acids or alkane sulphonates ($R\text{-}COO\text{-}Na^+$; $R\text{-}SO_3\text{-}Na^+$; $R=C_{10}\text{-}C_{12}$ alkyl). In aqueous solution they dissociate to a large anion, responsible for the strong detergent properties, and a small cation (Paulus, 1993). Their antimicrobial effect is restricted mainly to Gram-positive bacteria. Their point of attack is apparently the microbial cell membrane. Acid formulations of anionic surfactants are used as disinfectants in the dairy, beverage and food processing industries, in institutions and homes (Hugo and Russell, 1982; Paulus, 1993). Figure 3.8 presents the molecular structure of SDS.

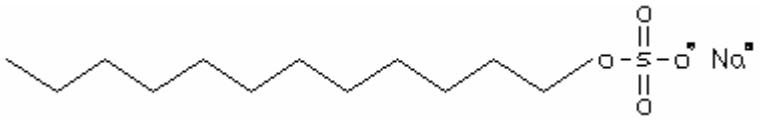


Figure 3.8 Chemical structure of sodium dodecyl sulfate.

Cationic surfactants, as CTAB (Figure 3.9), possess strong bactericidal, but weak detergent, properties. Cationic detergent usually signifies quaternary ammonium compounds – QAC´s (McDonnell and Russell, 1999). The antimicrobial properties of QAC´s depend on their structure and size, but specially on the length of the long-chain alkyl group. QAC´s bearing the C_{14} alkyl group exhibit maximum antimicrobial activity. The efficacy of QAC´s increases with temperature and pH (Paulus, 1993). They have a wide application ranging from the clinical to industrial purposes (Hugo and Russell, 1982; Paulus, 1993; Rossmoore, 1995; McDonnell and Russell, 1999). Figure 3.9 presents the molecular structure of CTAB.

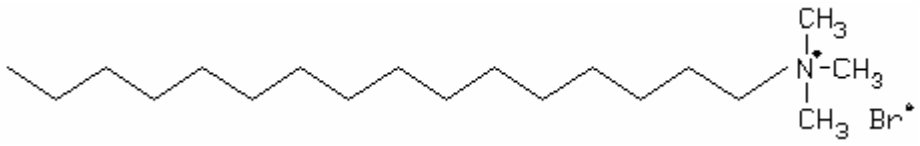


Figure 3.9 Chemical structure of cetyltrimethyl ammonium bromide.

Oxidizing biocides:

Sodium hydroxide (NaOH) purchased from Merck (Cat. No. 106467).

Sodium hypochlorite (SHC) purchased from Merck (13 % active chlorine; Cat. No. 105614).

Oxidizing agents have been widely used as disinfectants (Kim *et al.,* 2002), being disinfection by chlorine compounds gained wide acceptance commercially, probably because of its simplicity to use and its moderate costs; despite the major problem of secondary harmful products generated by this compounds (Hassen *et al.,* 2000; Kim *et al.,* 2002). The antimicrobial effects of oxidizing agents are attributed to their strong oxidation properties (Paulus, 1993).

The concentrations of each product tested were obtained by preparation with sterile distilled water.

3.5.1 Neutralization of the antimicrobial agents

After the contact of the chemical agent with the planktonic cells and with the cells within biofilm, they were subjected to a process of neutralization in order to quench their antimicrobial activity. The aldehyde-based biocides were neutralized by the dilution method, by which the chemical was diluted to a sub-inhibitory level, according to Johnston *et al.,* (1999) or with sodium bisulphite (Sigma) at a final concentration of 0.5 % (w/v). The surfactants were chemically neutralized by the following solution: (w/v) 0.1 % peptone, 0.5 % Tween 80 (Sigma) and 0.07 % lecithin (Sigma), dissolved in phosphate buffer pH 7. A concentrated neutralization solution was prepared and autoclaved prior to utilization.

3.6 Biofilm manipulation and analytical methods

3.6.1 Scrapping and disaggregation of the biofilms

Before and after each chemical treatment and in the defined sampling times, the biofilms that covered the stainless steel slides were completely scraped from the metal slides, using a metal scrapper, resuspended into 10 ml phosphate buffer, pH 7, and homogenised in a vortex (Heidolph, model Reax top) for 30 s with 100 % power input and used for further analysis. The efficiency of the process of biofilm scrapping and disaggregation was ascertained by staining the stainless steel plate, after the process, with 4`, 6-diamidino-2-phenylindole (DAPI). DAPI is believed to be very specific for DNA and is, thus, used to count total (including viable and non-viable) bacteria (Saby *et al.,* 1997). In order to detect residual bacteria on the metal surface, 1 ml of a DAPI solution (0.5 µg/ml) was placed on the surface and left to stand on dark for 5 min. To observe bacteria on the surface, a Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination was used with a 100 X oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained preparations consisted of a 359 nm excitation filter in combination with a 461 nm emission filter. A minimum of 20 microscopic fields were observed for each stainless steel surface inspected.

It was found that only about 1 – 2 % of the total number of bacteria adhered to the stainless steel surface was not efficiently removed with the procedure applied (Figure 3.10).

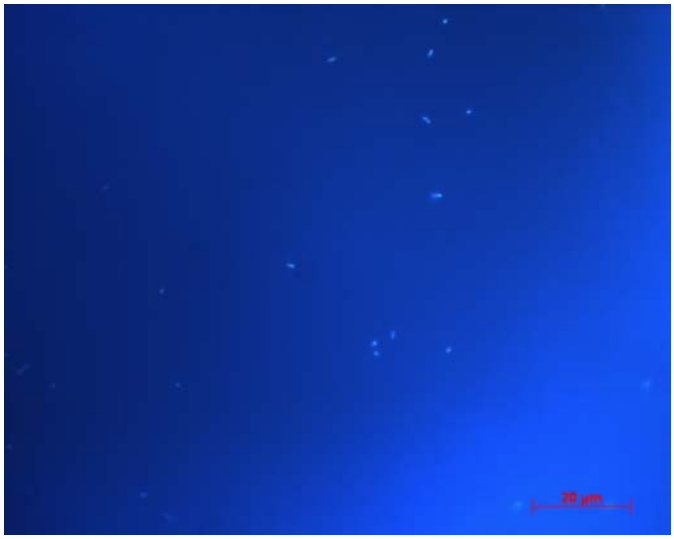


Figure 3.10 Representative microphotograph of a stainless steel surface after the process of scrapping and disaggregation of the biofilm. X 1320 magnification, bar =20 μm.

3.6.2 Extraction of the extracellular polymeric substances

The extraction of the extracellular components of the biofilm and the planktonic cells was carried out using Dowex resin (50X 8, NA^+ form, 20-50 mesh, Aldrich-Fluka 44445) according to the procedure described by Frølund *et al.* (1996). Briefly, it consists in resuspend of the biological samples (sessile and planktonic cells) in 20 ml of extraction buffer and to add 50 g of Dowex resin *per* g of volatile solids. The extraction took place at 400 min^{-1} for 4 h at 4 ºC. The extracellular components were separated from the cells with a centrifugation step (B. Braun, model Sigma 4K10) at 3777 *g* for 5 min. Prior to the extraction, the Dowex resin was washed with extraction buffer (2 mM Na_3PO_4, 2 mM NaH_2PO_4, 9 mM NaCl and 1 mM KCl, pH 7).

3.6.3 Biochemical analysis of the cells and biofilms

The biochemical characterisation of the planktonic cells and biofilms was performed by the determination of the proteins and polysaccharides quantification. The proteins were determined using the Lowry modified method (Sigma-protein assay kit nº P5656), with bovine serum albumin as a protein standard and the optical density (OD) values recorded at 740 nm. The polysaccharides were determined by the phenol-sulphuric acid method of Dubois *et al*, (1956), with glucose as standard and the OD values recorded at 490 nm.

3.6.4 Cell number quantification

To quantify the number of cells adhered to a surface or retained in a membrane, *P. fluorescens* were stained with the suitable stain and observed under epifluorescence microscopy. To observe the stained bacteria, a Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination was used with a 100 X oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained preparations was selected according to the stain used. The micrographs were obtained using a microscope camera (AxioCam HRC, Carl Zeiss). A program path (AxioVision, Carl Zeiss Vision) involving image acquisition and image processing was used to obtain the images.

A program path (Sigma Scan Pro 5, Sigma) involving object measurement and data output was used to obtain the number of cells.

The number of cells was estimated from counts of a minimum of 20 microscopic fields, for each analysis.

3.6.5 Biological mass quantification

The dry mass of the biological samples was assessed by the determination of the total volatile solids (TVS) of the homogenised bacterial and biofilm suspensions, according to the APHA, AWWA, WPCF Standard Methods (1989), method number 2490 A-D. The results were expressed in g of biofilm *per* cm^2 of surface area of the slide ($g_{biofilm}/cm^2$) in the case of biofilms or g of bacteria *per* unit of volume ($g_{bacteria}/l$) in the case of suspended cultures.

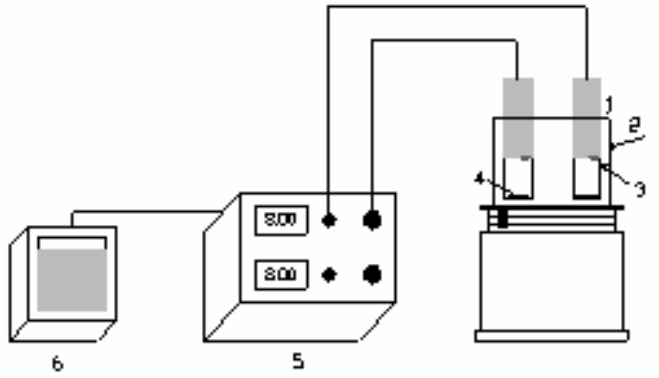
In some biofilm experiments, the results related with loss of biofilm mass are presented as percentage of removal, where the percentage of the biofilm removal was determined through the following equation:

$$Biofilm\ removal\ (\%) = [(W_0 - W_1)/W_0] \times 100 \qquad (3.1)$$

Where W_0 is the biofilm mass without chemical treatment ($g_{biofilm}/cm^2$) and W_1 is the biofilm mass after chemical treatment ($g_{biofilm}/cm^2$).

3.6.6 Metabolic activity - respiratory activity

The respiratory activity of the several biological (biofilm suspensions or suspended cultures) samples was evaluated by measuring oxygen uptake rates in a biological oxygen monitor (BOM) in short-term assays. The assays were performed in a Yellow Springs Instruments BOM (Model 53) and the procedure used was described elsewhere (Pereira *et al*., 2002a). Before each respirometry assay, the samples were carefully washed three times with phosphate-buffered saline solution (NaCl 0.85 %), resuspended in 10 ml of phosphate buffer pH 7 and placed in the temperature-controlled vessel of the BOM (T= 27 ºC ± 1ºC).



1 – Oxygen electrode
2 – Respirometry chamber
3 – Respirometry cell
4 – Magnetic stirrer
5 – Oxygen probe
6 – Data acquisition (computer)

Figure 3.11 Schematic representation of a biological oxygen monitor.

The temperature-controlled vessel of the BOM contained a dissolved oxygen (DO) probe connected to a DO meter. Once inside the vessel, the samples were aerated for ½ h to ensure the oxygen saturation and the consumption of any residual carbon source. The vessel was closed and the decrease of the oxygen concentration was monitored over time. The initial linear decrease observed (1) corresponds to the endogenous respiration rate. To determine the oxygen uptake due to substrate oxidation, a small volume (50 µl) of a glucose solution (100 mg/l) was injected within each vessel (point I). The slope of the initial linear decrease in the DO concentration, after glucose injection (2), corresponds to the total respiration rate. The difference between the two respiration rates gives the specific oxygen uptake rate due to the glucose oxidation, herewith referred as "respiratory activity", and expressed in mg of O_2 consumed *per* g of biofilm and *per* time (mg O_2/g_{biofilm} min), in the case of the biofilm solutions, and in mg of O_2 consumed *per* g of bacteria and *per* time (mg O_2/g_{bacteria} min), in the case of the suspended bacterial cultures.

In same cases, the results related with respiratory activity are presented as percentage of inactivation, where the decrease in the biofilm/bacterial activity obtained due to the

application of the chemical agents to the cells was determined as the difference between the respiratory activities of the samples before (control) and immediately after the treatment with chemical agent, and expressed as the percentage of inactivation according to the following equation:

$$Inactivation\ (\%) = [(A_0-A_1)/A_0] \times 100 \qquad (3.2)$$

where A_0 is the respiratory activity of the control assay, *i.e.*, without chemical treatment, and A_1 is the respiratory activity immediately after the application of each chemical concentration.

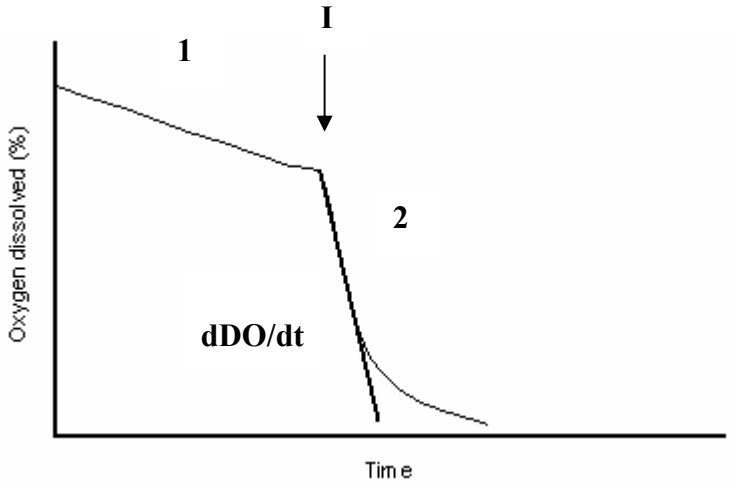


Figure 3.12 Typical profile of oxygen uptake in an assay of respiratory activity.

1 - Oxygen consumption due to endogenous metabolism;
I – Nutrient addition;
2 – Oxygen consumption due total metabolism (endogenous and exogenous);
dDO/dt - Slope of the initial linear decrease in the DO concentration after nutrient addition.

The respiratory activity was evaluated by means of the determination of the oxygen uptake rate due to glucose oxidation which, according to Stewart *et al.* (1994) and McFeters *et al.* (1995), may be more accurate than the traditional method of colony formation on agar media. This latter method has received much criticism specially when used to evaluate the effect of the antimicrobial agents (MacDonald *et al*., 2000). In fact, the method may underestimate the actual viable population since the bacteria on the biofilm can continue viable after the chemical application but may not grow on solid media. Also, due to the structure of biofilms and the difficulty to disaggregate cells within biofilms the method of culturability seems to be not appraisable to characterize a bacterial population in terms of viability.

3.6.7 Adenosine triphosphate measurement

The adenosine triphosphate (ATP) released from the cells was measured with the firefly luciferin-luciferase System/Sigma FL-AAM. The assessment of the cellular ATP with firefly luciferase catalyzes the reaction of luciferin with ATP to form an intermediate luciferyl adenylate, whose subsequent oxidation results in light emission with a quantum yield of about 90%. The integrated light intensity is directly proportional to the amount of ATP (Venkateswaran *et al.,* 2003).

In order to measure the ATP released after the required contact time with the chemical agent, 100 µl of the cellular suspension was added to 100 µl of a 25 fold dilution mixture of luciferine and luciferase. The light transmission was measured in a bioluminometer (Lumac, Biocounter M 25000) and the output values were recorded in relative light units (RLU).

Control experiments were made with phosphate buffer and with the different chemicals at the different concentrations tested.

3.6.8 Characterization of the proteins of the outer membrane

3.6.8.1 Outer membrane proteins isolation

The outer membrane proteins (OMP) were isolated according to the method described by Winder *et al.* (2000). Briefly, cells were harvested by centrifugation (3777 *g*, 5 min, 4 ºC). The resulting pellet was resuspended in 25 mmol/l Tris and 1 mmol/l $MgCl_2$ buffer (pH = 7.4). The solution was sonicated for 2 min (Vibracell, 60W) on ice to promote cell lysis. After sonication the solution was centrifuged (7000 *g*, 10 min, 4 ºC) in order to remove non-lysed cells. The supernatant was collected and *N*-lauroylsarcosine (Sigma) was added to give a final concentration of 2 % (w/v) in order to solubilize the inner membrane proteins and left on ice for ½ h. Afterwards the solution was centrifuged (17000 *g*, 1 h, 4 ºC) to recover the OMP. The pellet containing the OMP was resuspended in deionised water (1 ml) and stored at -20 ± 2 ºC until required.

3.6.8.2 Outer membrane proteins quantification

The protein content of the samples was determined using the Bicinchoninic Acid Protein Assay Kit (BCA) (BCA - PIERCE Cat. No. 23225) with BSA as standard.

3.6.8.3 Sample preparation

To a volume of 15 µl of OMP sample it was added 5 µl of sample buffer (25 mM Tris-HCl pH 6.8; 10 % (w/v) β-mercaptoetanol; 10 % (w/v) SDS; 0.1 % (w/v) bromophenol blue and 30 % (v/v) glycerol). This mixture was immersed in water, at 100 ºC, for 5 min, in order to promote protein denaturing, and immediately putt in ice. To each well of the gel it was added 15 µl of the sample resulting from the protein denaturing process.

Electrophoresis was performed at a constant current of 10 mA.

3.6.8.4 Outer membrane proteins analysis

The OMP samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as reported by Laemmli (1970), with 12 % (w/v) acrylamide.

The polyacrylamide gel electrophoresis separates molecules in complex mixtures according to size and charge. During electrophoresis there is an intricate interaction of samples, gel, matrix buffer, and electric current resulting in separate bands on individual molecules. Hence, the variables that must be considered in electrophoresis are gel pore size, gel buffer systems, and the properties of the molecule of interest.

The Laemmli buffer system is a discontinuous buffer system that incorporates sodium dodecyl sulfate in the buffer. In this system, proteins are denatured by heating them in buffer containing SDS and a thiol reducing agent (2-mercaptoethanol). The resultant polypeptides take on a rod-like shape and a uniform charged-mass ratio proportional to their molecular weights. Proteins are separated according to their molecular weight, making this system extremely useful for calculating molecular weights.

In Table 3.1 are listed the volumes of the different constituents required to completely fill two 12 % polyacrylamide gel cassettes.

Table 3.1 Volumes required to prepared two polyacrylamide gels at 12 %

Gel *	Distilled deionised water (ml)	30 % Degassed acrylamide/Bis (ml)	Gel buffer (ml)	10 % (w/v) SDS (ml)
R	5.10	6.00	3.75[1]	0.15
S	3.40	4.00	2.50[2]	0.10

* R – Gel resolving; S- Gel stacking

[1] Resolving gel buffer – 1.5 M Tris-HCl, pH 8.8

[2] Stacking gel buffer – 0.5 M Tris-HCl, pH 6.8

The monomer solution was prepared by mixing all the reagents and degas the mixture for 15 min. Immediately prior to pouring the gel in the electrophoresis system, was added for the resolving gel 90 µl of ammonium persulfate (APS) 10 % (w/v) and 9 µl of TEMED. For the stacking gel were added 60 µl of PSA and 6 µL of TEMED. The solutions were swirl gently to initiate polymerization. The electrophoresis gels were completely submersed with TGS buffer.

3.6.8.5 Staining with coomassie blue and silver nitrate

After electrophoresis the gels were removed from the gel cassette sandwich by gently separating the two plates of the gel cassette. After that, the gels were putt in a coomassie blue solution (50 % (v/v) methanol; 10 % (v/v) acetic acid; 0.05 % (w/v) coomassie brilliant blue; 40 % distilled water) for ½ h. After this time, the coomassie blue solution was replaced by a decolouring solution (30 % (v/v) methanol; 10 % (v/v) acetic acid; 60 % distilled water) during 5 min. The decolouring solution was replaced by a new solution. The gels were left in the solution for 16 h under agitation (80 min^{-1}). Afterwards the gels were placed in distilled water for 10 min before being silver stained.

In order to perform silver staining the gels were putt in sodium tiosulphate (0.2 g/l) during 1 min. The sodium tiosulphate was withdrawn and the gels were washed twice with distilled water for a period of 20 s and, then, the gels were left to react with silver nitrate (2 g/l) during ½ h. The silver nitrate was collected and the gels were washed with distilled water for 10 s. After this step the gels were allowed to contact with a solution of revelation (0.7 ml/l formaldehyde 37 % (v/v); 30 g/l potassium carbonate anhydride; 10 mg/l sodium

tiosulphate) for 5 min. At the end of the staining process the gels were putt in a solution of 50 g/l Tris and 2.5 % (v/v) acetic acid for 1 min.

The protein profiles obtained by SDS-PAGE sizes were determined by comparison with a molecular weight standard (161-0362 - Precision Protein Standards Unstained Range - BioRad). All the compounds used for the OMP analysis, sample preparation and staining of the gels were purchased from BioRad.

3.6.9 Polymerase chain reaction experiments

3.6.9.1 DNA extraction

The DNA extraction procedure was followed according to Yokoigawa *et al.* (1999). The cells were added to 100 µl of lysis buffer consisting of 10 % (w/v) NaCl (Merck), 2 % (v/v) Tween-20 (Merck) and 2 mM EDTA (Merck). The suspensions were then treated at 100 ºC on a heating block, for 10 min and centrifuged (3777 *g*, 5 min). Supernatant fluids were then used directly as polymerase chain reaction (PCR) templates.

3.6.9.2 Polymerase chain reaction amplification

PCR is a rapid procedure for *in vitro* enzymatic amplification of a specific segment of DNA. To perform a PCR, the DNA to be amplified is denatured by heating the samples. In the presence of DNA polymerase and excess of deoxyribonucleoside triphosphates (dNTP´s), oligonucleotides that hybridize specifically to the target sequence can prime new DNA synthesis. The first cycle is characterized by a product of indeterminate length; however, the second cycle produces the discrete short product which accumulates exponentially with each successive round of amplification. This can lead to many million-fold amplification of the discrete fragment over the course of 20 to 30 cycles.

There are three nucleic acid segments: the segment of double-stranded DNA to be amplified and two single-stranded oligonucleotide primers flanking it. Additionally, there is a protein component (DNA polymerase), appropriate deoxyribonucleoside triphosphates (dNTP´s). The primers are added in vast excess compared to the DNA to be amplified. They hybridize to opposite strands of the DNA and are oriented with their 3` ends facing each other so that synthesis by DNA polymerase (which catalyzes strands 5`- 3`) extends across the segment of DNA between them. One round of synthesis results in new strands of

indeterminate length which, like the parental strands, can hybridize to the primers upon denaturation and annealing. These products accumulate only arithmetically with each subsequent cycle of denaturation, annealing to primers, and synthesis. The second cycle of denaturation, annealing, and synthesis produces two single-stranded products that together compose a discrete double-stranded product which is exactly the length between the primer ends. Each strand of this discrete product is complementary to one of the two primers and can therefore participate as a template in subsequent cycles. The amount of this product doubles with every subsequent cycle of synthesis, denaturation, and annealing, accumulating exponentially so that 30 cycle should result in 2^{28} fold (270 million-fold) amplification of the discrete product (Ausubel *et al.,* 1999).

The objective of this step was to identify possible changes in the DNA at the species level by DNA fingerprinting using the primer named T3B (5`-AGGTCGCGGGTTCGAATCC-3`), described by McClelland *et al.* (1992).

The extracted DNA samples were diluted 1:10 in nanopure water.

The reaction was performed in 0.2 ml tubes by using a Biometra (Uno II) thermal cycler (Model Uno II). In all cases, reactions were carried out using 0.2 µl of native *Taq* DNA polymerase-5U/µl (Fermentas), 0.5 µl of T3B primer at 25 pmol (MWG AG Biotech.), 18.8 µl of nanopure water, 1.5 µl of $MgCl_2$ (25 mM), 0.5 µl of dNTP at 10 mM (Fermentas), 2.5 µl of amplification buffer (purchased with the *Taq* DNA polymerase) and 1 µl of extracted sample. The thermal program was as follows: 95 º (10 min – denaturation step) followed by 32 thermal cycles of 95 ºC (30 s), 50 ºC (30 s) and 72 ºC (60 s), corresponding, respectively, to heat denaturation of double-stranded target DNA; cooling to allow hybridization of specific primers to target DNA; primer extension by the action of DNA polymerase. The final cycle incorporated an 8 min chain elongation step (72 ºC). A 10 µl aliquot of each PCR product were visualized by 0.5 µg/ml ethidium bromide (Sigma) staining after electrophoresis (90 min at 70 V) through a 1.2 % agarose gel (BioRad) and their sizes were compared with a molecular weight standard (1 kb plus DNA ladder; GibcoBRL). The DNA fragments were visualized on a short-wave ultraviolet transilluminator Eagle-Eye II (Stratagene).

3.6.10 Scanning electron microscopy observations

The scanning electron microscopy (SEM) inspections always comprised the observation of at least 15 fields of each biofilm-covered slide. Prior to SEM observations, the biofilm samples were gradually (15 min each in 10, 25, 40, 50, 70, 80, 90 and 100 % v/v) dehydrated in an absolute ethanol (Merck) series to 100 % (v/v), and dried in a desiccator for 3 d. The samples were sputter-coated with gold and examined with a Leica S360 scanning electron microscope at 10-15 kV. The slides were not fixed because fixation procedures involves the use of chemicals that tend to react with some of the components at the biological matrix, as documented by Azeredo *et al.* (1999), hence modifying the real biofilm structure. SEM observations were documented through the acquisition of representative microphotographs.

3.6.11 Statistical analysis

The process of data analysis took place in two phases:

- exploration and description of the data;
- confirmatory statistical analysis.

For that purpose the statistics software package SPSS (version 11.5) was used to carry out the statistical tests (Kinnear, 1999).

The aim of the statistical treatment was to establish that a result is robust to repetition (or replication) of the study. For every experiment, the average and standard deviation (SD) were calculated as measures of central tendency and dispersion of the values acquired with the experiment. However, the screening and rejection of atypical values (outliers) was always done.

For confirmatory statistical analysis was used the paired samples Student *t*-test, in order to ascertain the significance of a difference between two means. The model underlying a *t*-test assumes that the data have been derived from normal distributions with equal variance, being considered a parametric test. The homogeneity of variance was assessed by the Levene´s test for equality of variance.

When there were serious violations of the assumptions of the Student *t*-test, non-parametric tests were used – Wilcoxon matched pairs test. Non-parametric tests do not carry specific assumptions about population distributions and variance.

The output of the statistical test is the *P* - value. When the *P* - value of a statistic was less than the significance level, the assumption of the statistic was said to be significant. If the *P* - value was larger than the significance level, the assumption was accepted.

The statistical calculations were based on a significance level equal or higher than 95 %.

Chapter 4

Validation of respirometry as a short-term method to assess the efficacy of biocides

Abstract

This chapter comprises the comparison of a short-term bacterial respiratory (metabolic) measurement based on the rate of oxygen uptake needed to oxidise glucose, the determination of viable and nonviable cells using Live/Dead® *Bac*Light™ kit and colony formation units, as indicators of the biocidal efficacy of OPA against *P. fluorescens*. Moreover, this chapter validates the respirometric method as an indicator of the cellular metabolic state.

4.1 Introduction

The disinfection efficacy of antimicrobial agents and the subsequent assessment of the microbiological metabolic state of the microorganisms can be evaluated by a variety of methodological approaches. The assessment of the efficacy of an antimicrobial agent is very important since misleading results may give rise to inefficient disinfection protocols. Traditionally, for heterotrophic bacteria, biocide efficacy has been evaluated by colony count enumeration (Stewart *et al.,* 1994). However, bacteria recovered on the solid media only represent culturable bacteria that are able to initiate cell division on that medium (Boulos *et al.,* 1999) whilst viable bacteria without the ability to grow on solid media are not accounted for. In addition, the culture method cannot be used accurately to evaluate the effect of chemicals on biofilms. Consequently, several other methods have been proposed as alternatives to the plate count method such as bioluminescence, impedimetry and respiratory activity (Stewart *et al.,* 1994). Respiratory activity can be determined by assessing the reduction of redox stains CTC (5-cyano-2, 3-ditolyl tetrazolium chloride), INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride) (Smith and McFeters, 1997; Hatzinger *et al.,* 2003) and XTT (benzenesulfonic acid hydrate) (Hatzinger *et al.,* 2003), followed by observation of the cells using direct microscopy and evaluation of the glucose consumption rate (Pereira and Vieira, 2001). Other methods such as the application of a specific or a combination of fluorochromes, with the use of direct microscopy (Boulos *et al.,* 1999; Terzieva *et al.,* 1996) and flow cytometry (Terzieva *et al.,* 1996; Virta *et al.,* 1998) have also been proposed to enumerate viable bacteria. More recently, molecular marker systems and detection methods have been applied to identify and quantify specific microorganisms as well as provide an indication of their viability (Yoshinori *et al.,* 2002).

Respiratory activity measured by oxygen uptake rate due to glucose oxidation has already been used to assess the potential of antimicrobial agents against planktonic bacteria and biofilms (Pereira and Vieira, 2001). However, no reports are available concerning the advantages and drawbacks of this technique. This study was designed to measure respiratory activity in order to assess the antimicrobial efficacy against *P. fluorescens* cells. The respiratory activity method using oxygen consumption rate due to glucose oxidation, the determination of viable and nonviable cells using Live/Dead® *Bac*Light™ kit and colony formation units on Plate Count Agar, were carried out as indicators of the microbiocidal

efficacy of OPA. Aditionally, this study is a challenge to demonstrate the reliability of respiratory activity as an indicator of the cellular state of the microorganisms since this methodology is fully used in the further experimental chapters.

4.2 Materials and methods

4.2.1 Microorganism

The microorganism used was *P. fluorescens* ATCC 13525 ^{T}.

4.2.2 Antimicrobial agent

OPA was used throughout this work.

4.2.3 Antimicrobial agent application

Periodically, aliquots of *P. fluorescens* were sampled from the 2 l reactor (sub-chapter 3.2.3), centrifuged (3777 *g*, 5 min) and washed three times with saline (NaCl 0.85 %) phosphate buffer pH 7. The pellets were resuspended in phosphate buffer (0.01 M) pH 7 in order to obtain a final bacterial suspension with an OD of 0.4 (λ = 640 nm). This bacterial culture was divided between several sterilised glass flasks that were placed in an orbital shaker (120 min^{-1}). The cell suspensions were exposed to several biocide concentrations (2, 7, 10, 15, 20, 25, 30, 35, 40, 45, 55, 65, 80 and 100 mg/l) for ½ h. After the required contact time, the biocide was neutralized as described below. From each bacterial suspension, several volumes were sampled in order to assess the metabolic state of the bacteria by respiratory activity, bacterial staining after microfiltration and agar plate cultivation.

4.2.4 Antimicrobial agent neutralization

As previously stated in sub-chapter 3.5.1, sodium bisulphite at a final concentration of 0.5 % (w/v) was added for 10 min (Walsh *et al.,* 1999a) to the bacterial cultures immediately after the ½ h biocide contact time.

4.2.5 Respiratory activity

The respiratory activity assays were performed in a Yellow Springs BOM (Model 53) according to the procedure described in sub-chapter 3.6.6.

4.2.6 Assessment of viability by a staining technique

The viability of *P. fluorescens* was assessed with L-7012 Live/Dead® (L/D) *Bac*Light™ Bacterial Viability kit developed by Molecular Probes Inc., using epifluorescence microscopy. This fast epifluorescence staining method was applied to estimate both viable and total counts of bacteria. *Bac*Light is composed of two nucleic acid-binding stains: SYTO 9™ and propidium iodide (PI). SYTO 9™ penetrates all bacterial membranes and stains the cells green, while propidium iodide only penetrates cells with damaged membranes, and the combination of the two stains produces red fluorescing cells.

After biocide treatment and neutralization, the various bacterial suspensions were diluted 1:10 and 300 µl of each diluted suspension were filtrated through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 µm) and stained with 250 µL diluted component A (SYTO 9) and 250 µl diluted component B (propidium iodide) for 15 min in the dark at 27 ± 1 ºC. The membrane was mounted in *Bac*Light mounting oil as described in the instructions provided by the manufacturer.

Dye solutions were prepared by dissolving 3 µl of component A in 1 ml of sterile-filtered (pore size 0.22 µm) distilled water and the same procedure was followed for component B. To observe the stained bacteria, a Zeiss (AXIOSKOP) microscope fitted with fluorescence illumination was used according to the procedure described in sub-chapter 3.6.4. The optical filter combination for optimal viewing of stained preparations consisted of a 480 to 500 nm excitation filter in combination with a 485 nm emission filter. The range of total cells for each field was between 50-200 cells/field.

4.2.7 Culturability method on Plate Count Agar

The selection of an adequate medium for the heterotrophic microbial grow is an important factor in the use of plate count method. In this work, tests were carried out in order to choose the appropriate medium. Plate Count Agar (PCA; Merck) was chosen since

it allowed small colonies to grow, preventing larger colonies from excessive growth through limiting diffusion of medium components. After biocide neutralization, the bacterial samples were diluted to the adequate cellular concentration in phosphate buffer. A volume of 30 µl of the bacterial diluted suspension were transferred onto PCA plates. Colony enumeration was carried out after 48 h at 27 ºC.

4.2.8 Calculations and statistical analysis

The loss of activity, viability and culturability due to biocide treatment, obtained by the different methods were calculated using the follow equations:

$$\text{Loss of activity (\%) (Respiratory activity)} = \frac{(\text{Exogenous respiratory activity control} - \text{Exogenous respiratory activity after biocide application})}{\text{Exogenous respiratory activity control}} \times 100 \quad (4.1)$$

$$\text{Loss of viability (\%) (}\textit{Bac}\text{Light counts)} = \frac{(\text{Total cells/ml control} - \text{Viable cells/ml after biocide application})}{\text{Total cells/ml control}} \times 100 \quad (4.2)$$

$$\text{Loss of culturability (\%) (Plate count)} = \frac{(\text{CFU/ml control} - \text{CFU/ml after biocide application})}{\text{CFU/ml control}} \times 100 \quad (4.3)$$

$$\text{Log reduction} = \text{Log (number of cells or CFU/ml control)} - \text{Log (number of cells or CFU/ml after biocide application)} \quad (4.4)$$

The MBC (minimum bactericidal concentration) for each method was determined as the lowest concentration of biocide where no activity, viability or culturability was detected according to Johnson *et al.* (2002).

Paired *t*-test analyses were performed to estimate whether or not there was a significant difference between the results obtained by the different methods. Statistical calculations were based on confidence level equal or higher than 95 %.

4.3 Results and discussion

The influence of OPA concentration on the activity, viability and culturability of *P. fluorescens* cells was evaluated through three different methods viz. respiratory activity, *Bac*Light viability kit and colony formation on plate count agar (culturability). Figures 4.1, 4.4 and 4.5 present, respectively, the values of the respiratory activity (exogenous and endogenous) *per* gram of cells, the number of viable cells *per* ml of bacterial suspension and the number of colony formation units *per* ml of bacterial suspension, as a function of OPA concentration.

4.3.1 Bacterial respiratory activity after antimicrobial agent application

The activity of the biocide (OPA) on the bacterial respiratory activity of *P. fluorescens*, evaluated by the measurement of the oxygen uptake rate due to glucose oxidation, gives the fraction of the cells that are active, since they exhibit metabolic activity (Figure 4.1).

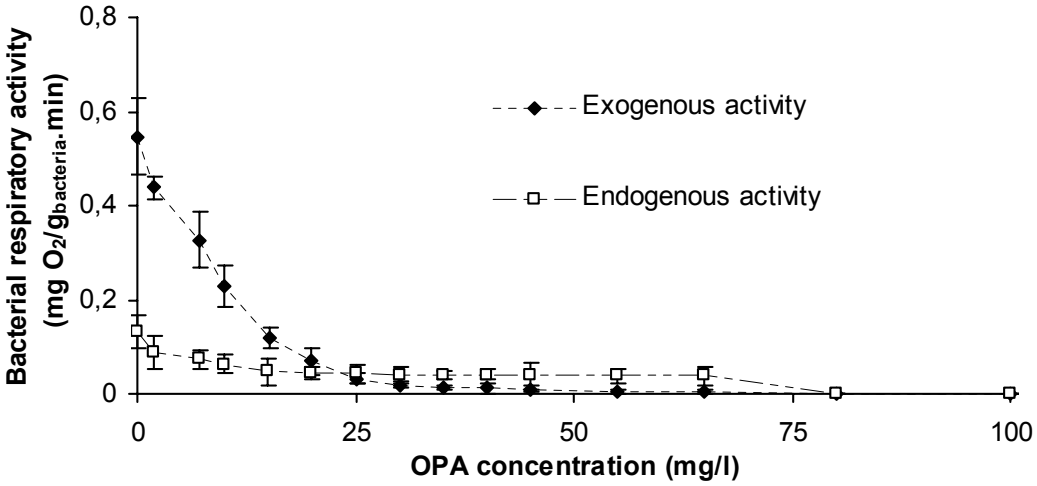


Figure 4.1 Exogenous and endogenous respiratory activity of *P. fluorescens* as function of OPA concentration.

The use of the respiratory activity showed that when bacteria were exposed to OPA concentrations higher than 80 mg/l bacterial activity was not detected when measured as the exogenous respiration rate. For OPA concentrations between 20 and 80 mg/l, a high decrease of exogenous respiratory activity was observed. For low OPA concentrations (< 20 mg/l), the decrease in activity was very sharp and dependent on OPA concentration.

Comparison of both rates showed that exogenous respiration rate is more dependent on biocide concentration than endogenous respiration rate. However, *P. fluorescens* retained considerable endogenous activity that remained more or less constant for a large range of OPA concentrations. Furthermore, the endogenous activity for OPA concentrations higher than 20 mg/l was even higher than the exogenous activity. These results suggest that even if the cells are not able to oxidise external substrates after contact with the biocide they may recover when the biocide is removed, since they maintain basal metabolism even in the presence of high biocide concentrations. The endogenous metabolism should be seen as a state where no net growth is observed. When bacteria are depleted of external substrates or faced with factors that cause stress, the cells often use intracellular materials as an energy source to maintain their survival (Russell and Cook, 1995). Several authors (Gilbert *et al.,* 1990; McDougald *et al.,* 1998) reported a similar effect to that found in this work, where bacteria can become exogenously dormant in response to unfavourable environmental conditions whilst still maintaining residual metabolism.

4.3.2 Bacterial viability after antimicrobial agent application

Figure 4.2 shows the number of *P. fluorescens* cells after OPA contact for ½ h exhibiting different colours due to *Bac*Light viability staining.

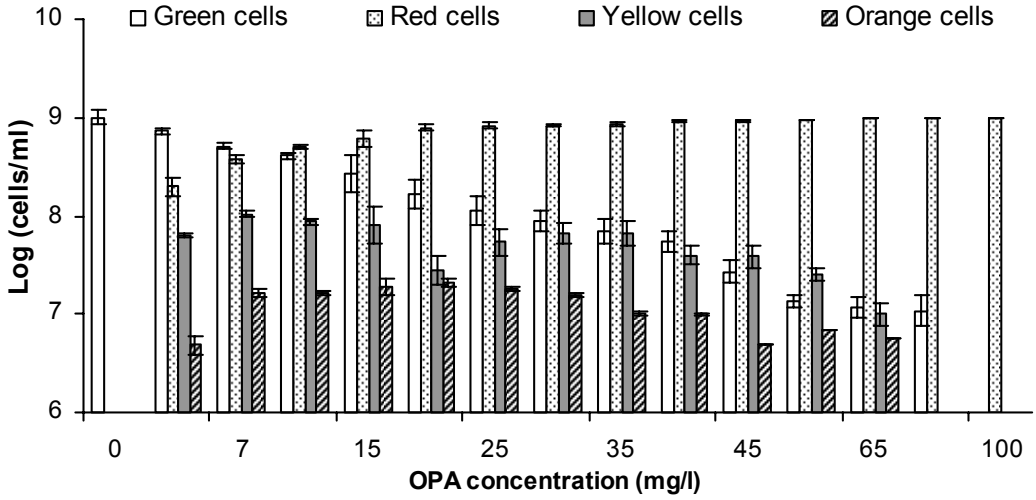


Figure 4.2 Effect of different OPA concentrations on *P. fluorescens* cells, examined by *Bac*Light stains.

According to Figure 4.2, for 80 mg/l and for 100 mg/l of OPA only red and green red cells could be found. The MBC determined using this method was 100 mg/l and for a

concentration of 80 mg/l of OPA, the log reduction was 1.8. When testing the intermediate range of OPA concentrations (between 2 mg/l and 65 mg/l) some green cells appeared yellowish and some red cells appeared orange, depending on biocide concentration (Figure 4.3). The colour differences between viable (green) and nonviable (red) bacteria were very easy to distinguish as can be seen in Figure 4.3.

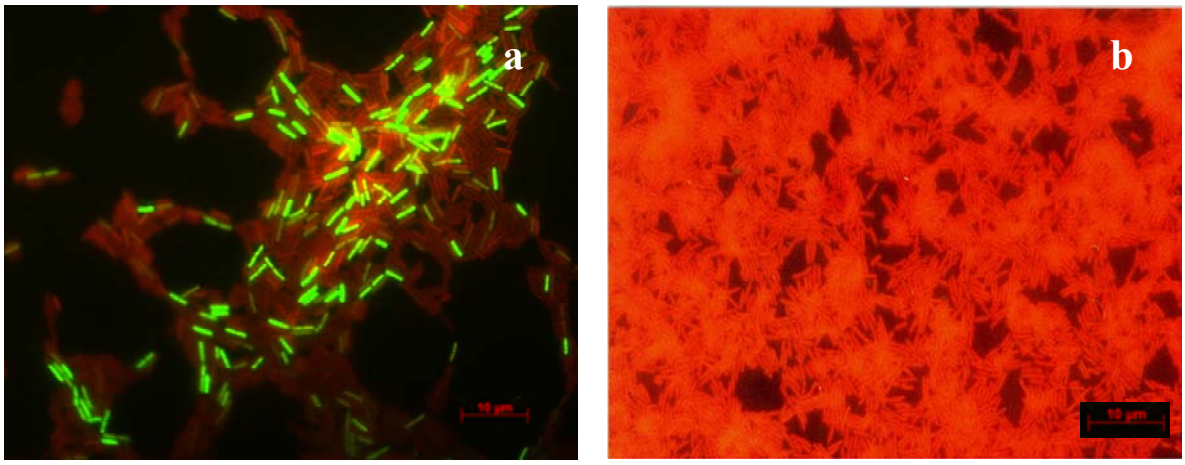


Figure 4.3 Epifluorescence photomicrograph of *P. fluorescens* planktonic cells after application of 55 (**a**) and 100 mg/l of OPA (**b**). X 1320 (**a**); X 1000 (**b**) magnification, bar = 10 μm.

Figure 4.4 displays the number of bacterial viable cells calculated according to different approaches: a) Boulos *et al.* (1999) consider cells fluorescing green and yellow as viable and cells fluorescing red and orange as nonviable; b) the manufacturer of the *Bac*Light kit considers green cells as viable and red, yellow and orange cells as nonviable.

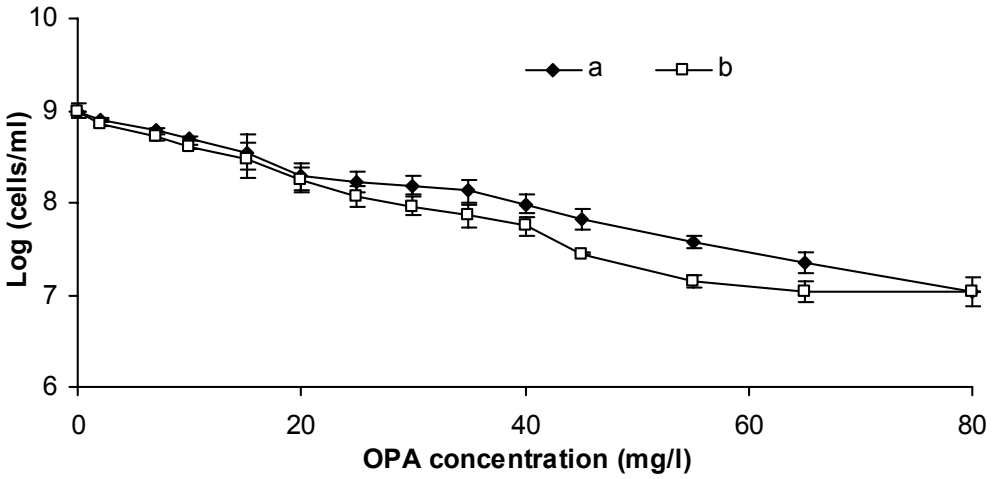


Figure 4.4 Number of viable cells obtained by Live/Dead® *Bac*Light™ Bacterial Viability kit, as function of OPA concentration. **a**) according to Boulos *et al.* (1999); **b**) according to the *Bac*Light kit manufacturer.

As expected, for both approaches, viable counts decreased with the increase of biocide concentration. However, in the above mentioned intermediate range of concentrations (between 2 mg/l and 65 mg/l), significant differences between the results obtained by each approach are noticeable.

The different range of colours found after biocide application and L/D *Bac*Light visualization (Figure 4.2 and Figure 4.3) is attributed to different metabolic states of the bacteria. Maukonen *et al.* (2000) studying the food borne bacteria *L. monocytogenes* and *P. fragi*, also reported the appearance of yellow and orange cells after staining with the *Bac*Light kit. They considered those cells injured, meaning that they could probably recover and reproduce after a period of time. Based on this argument, in the present work, the appearance of injured cells (Figure 4.2) was dependent on the OPA concentration, since an increase in the concentration lower than the lethal concentration (100 mg/l) gave rise to the appearance of a greater number of yellow and orange cells, while for the higher OPA concentrations (higher than the lethal concentration) only red cells were found.

4.3.3 Bacterial culturability after antimicrobial agent application

Figure 4.5 presents the total bacterial counts in terms of colony forming units (CFU) as function of OPA concentration.

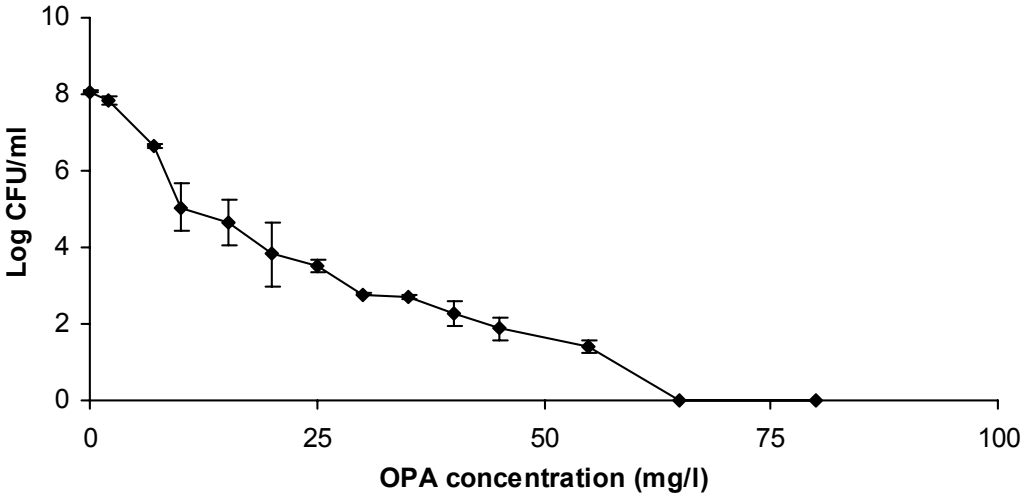


Figure 4.5 Total bacterial counts (colony forming units-CFU), as a function of OPA concentration.

The results obtained using the culture method showed that the MBC is 65 mg/l. For OPA concentration of 7 mg/l, the decrease of bacterial culturability corresponded to a log reduction of 1.4. However, a 5 log reduction on the number of CFU was registered for concentrations higher than 25 mg/l.

The number of viable cells (Figure 4.4) and culturable cells (Figure 4.5) before biocide treatment were not comparable (difference of about 1 log), which is probably related to the limitations of the plate count method. It has long been recognized that the single use of culture-based enumeration techniques may significantly underestimate the numbers of viable cells. Several reasons may account for this difference: i) presence of starved or injured cells or potentially viable but not culturable (VBNC) cells (Banning *et al.,* 2002) that are not able to initiate cell division at a sufficient rate to form colonies; ii) inadequate culture conditions; iii) aggregation of bacteria that can lead to the formation of one colony from more than one cell, thereby underestimating the total number of cells. Ericsson *et al.* (2000) also considered that the method of cultivation on a solid is often inadequate due to the failure of the bacterial cell to reproduce on standard nutrient agar plates. Nevertheless, this may not mean that the cells were nonviable. In fact, cells can be viable but lack the ability to divide. It can also be argued that the viability method based on *Bac*Light can overestimate the number of viable cells as appeared to be suggested by the activity results. Ericsson *et al.* (2000) also showed that even cells that fluoresced green after staining with *Bac*Light, had different reproductive responses: cells could divide more than once, divide

only once or not show the ability to divide in a counting chamber. Those cells probably lost their potential to form colonies, but they remain physically intact and metabolic active or with a diminished activity. These arguments are reinforced with the discrepancy encountered between activity and viability results.

The inconsistency between the number of culturable and viable cells increases with the application of biocide. In fact, after biocide application, the comparison of Figures 4.2 and 4.4 with Figure 4.5 show that, for the same OPA concentration, higher numbers of viable bacteria are detected by the *Bac*Light kit than the culturable bacteria found on the PCA plates. This comparison indicates the probably existence of VBNC bacteria. The limitations of the plate count method can also be observed, for instance, for a biocide concentration of 55 mg/l, where *Bac*Light viable counts exceed the number of CFU by at least 5 orders of magnitude. So, CFU underestimate the actual viable population, since the bacteria exhibit activity and are viable after biocide application but are not able to grow on solid media as also reported by McDougald *et al.* (1998).

4.3.4 Comparison between bacterial respiratory activity, viability and culturability

For comparative purposes, the loss of bacterial activity, viability and culturability were calculated and are presented in Figure 4.6.

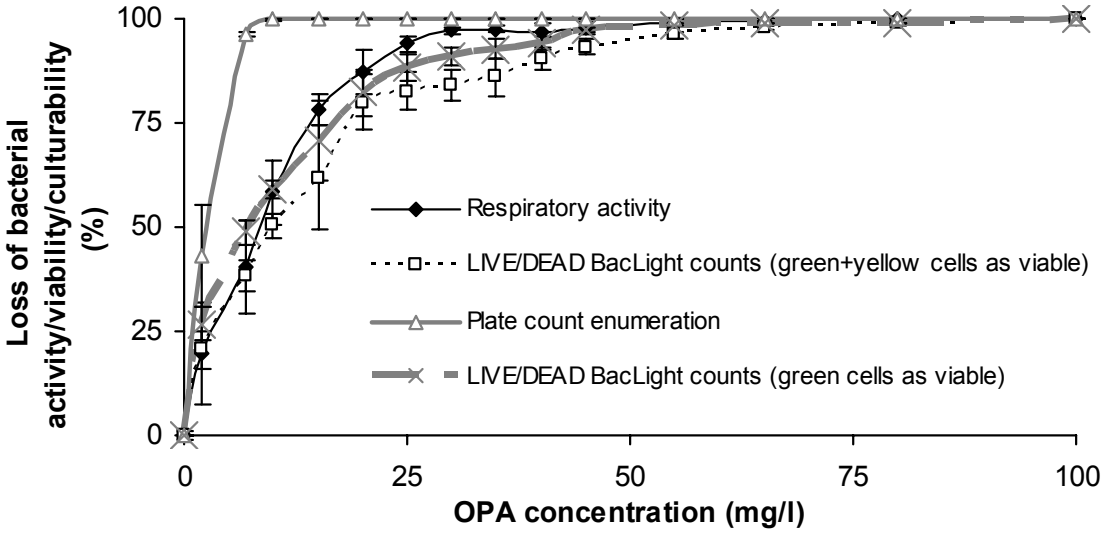


Figure 4.6 Loss of bacterial activity, viability and culturability assessed by respirometric activity (oxygen uptake rate – exogenous activity), Live/Dead® *Bac*Light™ bacterial viability kit and colony forming ability.

Figure 4.6 clearly shows that bacteria lose culturability and yet still exhibit clear signs of metabolic activity and viability. In fact, OPA induced a sharp decrease in culturability (CFU counts) that is more pronounced than the loss of bacterial activity and viability. For small OPA concentrations, cells lose culturability abruptly while maintaining activity and viability. It can be seen that the *Bac*Light viable counts, calculated using the above approaches, decreased much less than did the loss of activity monitored by respiratory activity. A loss of bacterial viability of about 90 % was obtained for a concentration of 40 mg/l of biocide (*Bac*Light kit) while a 90 % loss of activity was assessed for 20 mg/l of OPA, *i.e.*, the MBC to obtain a reduction of 90 % is 40 mg/l or 20 mg/l using the viability and activity assessments respectively. The overall results presented in Figure 4.6 show that a large number of bacteria, retained physiological and metabolic activity after biocide treatment, while most of them could not form colonies on conventional medium.

Using the data depicted in Figure 4.6, some correlations were made in order to evaluate the possibility of obtaining linear relationships between the various tested methods. Those correlations showed that the loss of activity assessed by respiratory activity and the loss of viability by *Bac*Light kit, counting yellow cells as viable, had a good correlation factor (R^2=0.974). Nevertheless, the results of the latter methods were not statistical equivalent ($P < 0.001$), suggesting the existence of significant differences in the efficacy of the biocide through assessment of bacterial viability using *Bac*Light viability kit and the respiratory activity method. Conversely, if the yellow cells were enumerated as nonviable, these methods were strong correlated with a R^2=0.982, and were not statistically different ($P > 0.10$).

The results obtained by *Bac*Light counts, considering the yellow cells as viable, and by the plate count method had a poor correlation factor of 0.433 ($P < 0.01$). Counting yellow cells as nonviable, the correlation factor R^2 increased to 0.637 but the methods were still statistical different $P < 0.01$. The linear adjustment of the loss of activity and the plate count results are correlated with a factor of 0.549 ($P < 0.02$).

As stated by Stewart *et al. (*1994), specific methods based on the physiological or metabolic activity supply more information on the action of the products used as biocides than the ability to grow and form colonies on a solid media. Later, McFeters *et al.* (1995) also showed that the determination of the oxygen uptake levels is more accurate than the traditional methods of bacterial enumeration by colony formation on agar media. These

plate count method may overestimate biocide efficacy since bacteria may remain viable after biocide application but may not grow on solid media. This loss of culturability led to the assumption that bacterial cells in this state were VBNC cells (McDougald *et al.,* 1998; Kawai *et al.,* 1999). Concerning the difference of the loss of viability assessed by *Bac*Light stains and respiratory activity (Figure 4.6) may be due to the amount of injured cells. Some of the bacteria that had no respiratory activity but fluoresced green and yellow, are probably in a stressed state, justifying the different MBC given by each method. In fact, even though some authors (Defives *et al.,* 1999; Ericsson *et al.,* 2000) advocate that the *Bac*Light viability kit avoids discrepancies in counts of total and viable bacteria, the methodology cannot discriminate the cellular status of the cells. The state of some of the total viable cells, detected by the *Bac*Light stains, could be seen as a transitory stage in the degeneration of the bacterial cells. However, it is not possible to quantify whether the SYTO 9™ dye could give false viable cells (Lehtinen *et al.,* 2004; Stocks, 2004) since the exogenous respiration results suggested that these cells may be dead. Braux *et al.* (1999) also reported a discrepancy effect in the results of oxidative stress caused by periacetic acid measured by respirometry using CTC, *Bac*Light viable counts and plate counts, respectively. No significant effect was observed with *Bac*Light viable counts, compared to the other methods. Their results suggested that respiratory activity and culturability are more rigorous criteria to assess the effect of chemical stress than membrane permeability assessed by PI uptake. Furthermore, the use of fluorescent dyes may have some drawbacks: dyes may be toxic to the bacteria at concentrations used in the assays and some viable bacteria may not incorporate the dye or may accumulate insufficient dye to become detectable *(*Hatzinger *et al., 2003);* moreover, after the application of fluorescent dyes the bacterial cells cannot be used in further studies. Conversely, with the respirometry based on oxygen uptake, subsequent analysis such as the reestablishment of respiration after biocide removal or inactivation (regrowth) can be carried out.

The results obtained with cells in suspension suggest that Live/Dead kit gives the most conservative estimate of biocidal efficacy and may be the most useful method. However, besides being a method that gives a higher MBC, it is expensive and time consuming. Furthermore, application of the *Bac*Light viability kit to determine the biocidal efficacy of OPA against biofilms proved to be inaccurate due cell aggregation promoted by EPS and to the reaction of the dyes with the EPS, as shown in Figure 4.7.

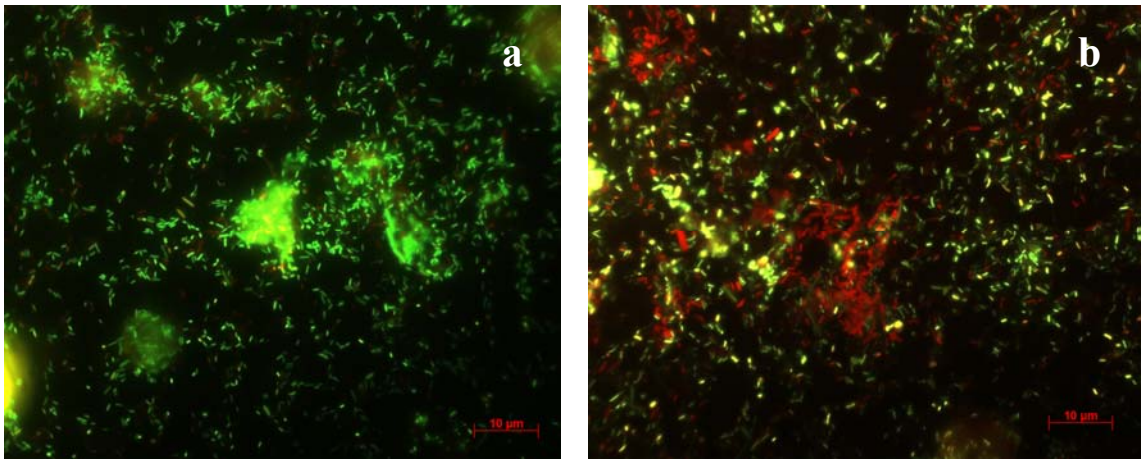


Figure 4.7 Epifluorescence photomicrograph of cells within biofilms formed by *P. fluorescens* before OPA treatment (**a**) and after application of 50 mg l^{-1} of OPA (**b**). X 1320 magnification, bar=10 μm.

The respiratory activity of *P. fluorescens* biofilms on stainless steel surfaces after treatment with biocides, has been reported previously (Pereira and Vieira, 2001). These assays were performed on biofilms immediately after exposure to the biocides and showed the high potential of the respiratory technique to assess efficacy against intact biofilms.

4.4 Conclusions

The use of different methods to evaluate biocide efficacy can lead to different conclusions regarding the effects caused by the biocide. Different MBC values were obtained depending on the method used to assess the efficacy of the biocide: 80 mg/l by respiratory method, 100 mg/l by the viability and 65 mg/l by the culturability method. Short-term respirometry proved to be a rapid, reliable, economic and easy methodology that can be used to evaluate biocide efficacy against aerobic, heterotrophic, carbon-consuming bacteria.

Chapter 5

Characterization of phenotypic changes in *Pseudomonas fluorescens* due to biofilm formation – effect of hydrodynamic conditions

Abstract

A comparison of the biochemical, metabolic and structural characteristics of *P. fluorescens* in planktonic state and developed in biofilms is presented in this study. The effects of the flow regime (turbulent and laminar) in those characteristics are also studied. The comparative study is performed by assessing the respiratory activity, the cell number, the amount of proteins and polysaccharides, the expression of outer membrane proteins and the analysis of the structural characteristics. The possibility of contamination in the process of biofilm development or the existence of cellular mutation is assessed by DNA analysis. For comparative purposes, biofilms formed under turbulent and laminar flow, are characterized in terms of mass formation ability, amount of total, extracellular and cellular proteins and polysaccharides and structural characteristics.

5.1 Introduction

The better understanding of biofilm behaviour is crucial since medical infections and biofouling of industrial components is often associated with biofilm formation (Stoodley *et al.,* 1999; Hall-Stoodley *et al.,* 2004). The occurrence of biofilms in these environments can lead to profound negative impacts and, consequently, to higher economical costs and risks in human health, as referred in Chapter 2. One of the earliest observations related to the different characteristics observed in planktonic cells and sessile cells is the increased resistance of biofilm cells to antimicrobial agents and other adverse environmental conditions (Costerton *et al.,* 1987; Brown and Gilbert, 1993). Phenotypic variation is a common phenomenon in Gram-negative bacteria that often involves environmentally regulated changes in surface components leading to alterations in observable phenotypes (Drenkard and Ausubel, 2002). In fact, a switch from planktonic state to growth in a biofilm form is believed to result in profound and complex phenotypic changes in bacteria (Davies *et al.,* 1998; Tolker-Nielsen *et al.,* 2000; Sauer and Camper, 2001; Svensäter *et al.,* 2001; Sauer *et al.,* 2002). Some reports on the properties of bacteria present in biofilms indicate that the growth on surfaces involve significant changes in gene transcription, including the establishment of new genetic traits (Davies *et al.,* 1995a; Christensen *et al.,* 1998; O´Toole and Kolter, 1998).

The analysis with molecular techniques of biofilm development and behaviour clearly demonstrate the influence of several genetically regulated factors. However, physical forces acting on the biofilm can also influence biofilm structure (Hall-Stoodley and Stoodley, 2002). One of the most important factors affecting biofilm structure and behaviour is the velocity field of the fluid in contact with the microbial layer (Vieira *et al.,* 1993; Stoodley *et al.,* 1999; Pereira *et al.,* 2002; Purevdorj *et al.,* 2002). The hydrodynamic conditions will determine the rate of transport of cells and nutrients to the surface, as well as the magnitude of shear forces acting on a developing biofilm.

The purpose of this work is to provide a phenotypic comparative study between *P. fluorescens* cells in planktonic and sessile state. The influence of the flow regime in the biofilm characteristics is also studied.

5.2 Materials and methods

5.2.1 Microorganism

The microorganism used was the *P. fluorescens* ATCC 13525 ^{T}.

5.2.2 Experiments with planktonic bacteria - microorganism growth

Periodically, aliquots of *P. fluorescens* suspended cultures, obtained according to sub-chapter 3.2.4, were sampled from the 2 l reactor, centrifuged (3777 *g*, 5 min), washed three times with saline (NaCl 0.85 %) phosphate buffer pH 7 and resuspended in phosphate buffer pH 7. These bacterial suspensions were immediately used to assess the cellular respiratory activity, being thereafter submitted to the EPS extraction, in order to determine the amount of total, extracellular and cellular proteins and polysaccharides, the cellular number, the mass of bacteria and the OMP expression.

5.2.3 Experiments with biofilms – biofilm set-up

Biofilms were formed by *P. fluorescens*, under turbulent and laminar flow, in flow cell reactors, according to the procedure described in sub-chapter 3.2.

5.2.4 Scrapping and disaggregation of the biofilms

The biofilm that covered the metal slides was completely scrapped from the metal slides (sub-chapter 3.6.1). The homogenised suspensions of biofilms were used to assess the respiratory activity of the biofilms and the biofilm mass.

5.2.5 Respiratory activity

The respiratory activity of the several biological samples was evaluated by measuring oxygen uptake rates in a BOM in short-term assays, according to the procedure described in the section 3.6.6, and thereafter were submitted to a process of EPS extraction (sub-chapter 3.6.2).

5.2.6 Biofilm and planktonic cells mass quantification

The dry planktonic cell mass and the biofilm cell mass were assessed both by the determination of the TVS following the procedure described in sub-chapter 3.6.5.

5.2.7 Quantification of the number of cells

After being separated from the extracellular products, the bacterial cells were diluted to an adequate concentration. Thereafter, the bacterial suspensions were microfiltrated through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm) and then stained with 400 μL of DAPI at 0.5 μg/ml and left on the dark for 5 min. The microscopic observation of the stained preparations with DAPI was performed as described in sub-chapter 3.6.1.

5.2.8 Biochemical analysis of the planktonic cells and biofilms

The biochemical characterisation of the planktonic cells, biofilm constituents and biofilms was performed by the determination of the total proteins and polysaccharides quantification according to the procedure described in sub-chapter 3.6.3.

5.2.9 Outer membrane proteins analysis

The OMP of the cells were subjected to a SDS-PAGE procedure (sub-chapter 3.6.8).

5.2.10 Polymerase chain reaction experiments

A volume of 20 μl of both planktonic cells and sessile cells, collected after the EPS extraction procedure, were subjected to PCR experiments, according to the procedure described in sub-chapter 3.6.9.

5.2.11 Scanning electron microscopy observations

During the experiments, several stainless steel slides covered with biofilms were observed by SEM following the method referred in sub-chapter 3.6.10.

5.3 Results and discussion

This work comprises two comparative studies: a comparison between planktonic cells and sessile cells obtained from biofilms formed under turbulent and laminar flow, and a comparison between biofilms formed under turbulent and laminar conditions.

5.3.1 Characterization of cells developed in planktonic state and within biofilms

The cellular characterization was assessed in terms of respiratory activity due to glucose oxidation, number of cells, protein and polysaccharide content, morphological characteristics and outer membrane protein expression.

Table 5.1 presents the cellular respiratory activity, number of cells *per* g of biological mass and the amount of total proteins and total polysaccharides *per* cell (planktonic cells and sessile cells obtained from the turbulent and laminar biofilms).

Table 5.1 Values of respiratory activity, number of cells, proteins and polysaccharides content of planktonic cells and sessile cells developed in turbulent and laminar biofilms. Mean ± SD

		Biofilm cells	
	Planktonic cells	**Turbulent**	**Laminar**
Respiratory activity ($mg\ O_2/g_{cells.min}$)	0.652 ± 0.19	0.400 ± 0.07	0.0788 ± 0.008
Number of cells ($cells/mg_{cells}$)	$1.19 \times 10^{11} \pm 2.0 \times 10^{10}$	$2.55 \times 10^{14} \pm 1.2 \times 10^{13}$	$1.12 \times 10^{12} \pm 1.0 \times 10^{11}$
Total proteins content (pg/cell)	879 ± 285	1.25 ± 0.38	6.69 ± 2.4
Total polysaccharides content (pg/cell)	2700 ± 1050	0.105 ± 0.026	9.30 ± 3.3

Phenotypic features as a consequence of the cellular state have significant impact on the design of biofilm control procedures, since current disinfection procedures are based on tests using cells in planktonic state (EN 1276, 1997; Wirtannen *et al.,* 2001). Besides several authors (Costerton *et al.,* 1995a; Tolker-Nielsen *et al.,* 2000; Sauer and Camper, 2001; Svensäter *et al.,* 2001; Sauer *et al.,* 2002) propose that biofilm formation trigger series of phenotypic changes, there are few studies providing quantitative evidences between cells in different states. Studies regarding the influence of the flow regime under which the biofilms are formed in the bacterial phenotype are even lesser.

According to Table 5.1, planktonic cells have a higher respiratory activity than the cells of both turbulent (about 2 times) and laminar (8 times) biofilms. Furthermore, turbulent biofilms were more active (5 times) than the laminar ones. In biofilms, the metabolic activity may reach an extent where biofilm bacteria are still viable, even if they do not show signs of viability as the capability to grow in a solid medium (Fux *et al.,* 2005a). So, this prolonged starvation induces loss of culturability whereas the cells remain metabolically active and structurally intact, being this viable but non-culturable state the main reason for the low detection rate of biofilm infections by routine culture (Fux *et al.,* 2005a). Consequently, as previously demonstrated in Chapter 4, when biofilms are the issue, the assessment of the respiratory activity due to oxygen uptake rate may be more accurate than the traditional method of colony formation on agar media to assess the viability of bacteria within biofilms (Stewart *et al.*, 1994; McFeters *et al.*, 1995).

Concerning the differences found for the metabolic activity of the cells within biofilms formed under different flow regimes, it is known that high shear stress can stimulate catabolic activity of biofilms (Liu and Tay, 2001). The catabolic activity is directly correlated with electron transport system activity. The respiratory activity of cells is coupled to the proton translocation activity and a clear linkage of O_2 reduction to proton translocation (Babcock and Wikstrom, 1992). Thus, the magnitude of catabolic activity would be proportionally related to the activity of proton translocation across cell membrane (Liu and Tay, 2001). Teo *et al.* (2000) revealed that proton translocation would induce the dehydration of cell surface, which could facilitate and strengthen the cell-cell interaction, and further lead to the creation of a high density of a microbial community. So, the catabolic activity of cells would play an important role in the development of a stronger biofilm (Liu and Tay, 2001). Also, this difference could be due to the existence of a higher number of cells within turbulent biofilms when comparing with the laminar ones.

The comparison of the number of cells *per* mg of biological mass reveals that, turbulent biofilms have a higher number of cells than the planktonic situation (about 2000 times). The same trend happens for laminar biofilms. Nevertheless, the difference is not as sharp as for the planktonic situation (about 200 times more). The cellular biochemical composition showed that planktonic cells have a pronounced higher content of proteins (about 700 times more and 130 times than, respectively, turbulent and laminar biofilms) and polysaccharides than biofilm cells (about 26000 times and 300 times than, respectively, turbulent and laminar biofilms), being this difference much more pronounced in the case of turbulent biofilms.

In order to observe if morphological changes occurred, the cells developed in the planktonic situation and in the biofilms, after the EPS extraction procedure, were stained with DAPI and observed with epifluorescence microscopy, as shown in Figure 5.1.

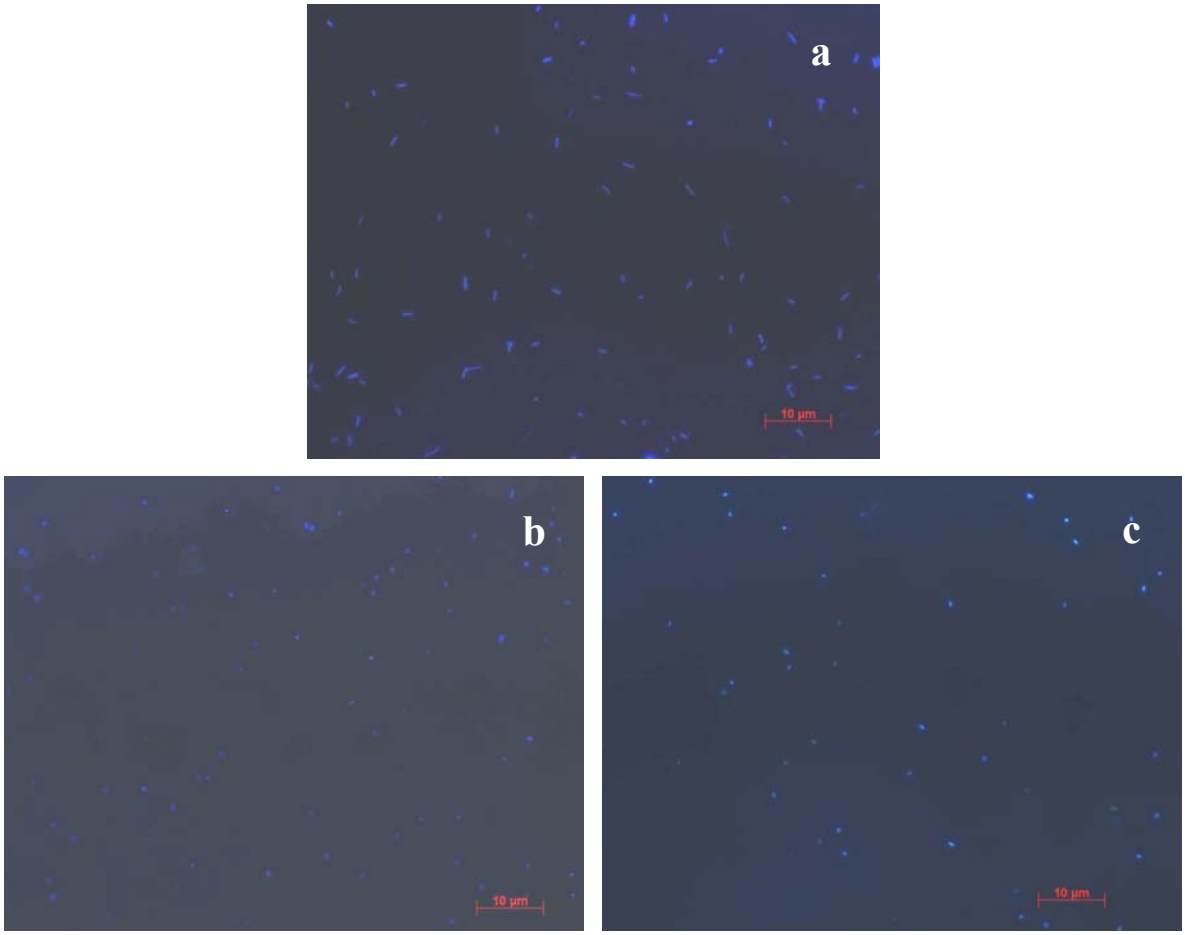


Figure 5.1 Epifluorescence photomicrograph of cells grown in planktonic state (**a**) and within biofilms formed under turbulent (**b**) and laminar (**c**) flow. X 1320 magnification, bar =10 µm.

From Figure 5.1 it can be observed that cell development in planktonic state and within turbulent and laminar biofilms resulted in morphologically changes. Planktonic cells have an elongated shape (rod shape), characteristic of *P. fluorescens* and the cells are bigger than in the sessile state. Cells within biofilms differ from the planktonic state as they appear to have a spherical shape. Concerning cells within biofilms formed under different flow regimes, cells from laminar biofilms seem to be bigger and to have a shape not as spherical as the cells from the turbulent biofilms. Arguably, this cellular feature could be related with the natural ability of bacteria to actively adapt to stress conditions. Additionally, this result corroborates the one found for the biochemical composition (Table 5.1), where the differences found in the proteins and polysaccharides content is certainly related with the differences existent in the bacterial cellular size, since planktonic cells were the ones that presented the more elongated cellular size, being, accordingly, the ones that presented the higher concentration of proteins and polysaccharides.

The OMP of planktonic cells and cells from turbulent and laminar biofilms were isolated and analysed by SDS-PAGE. The OMP profiles obtained are presented in Figure 5.2.

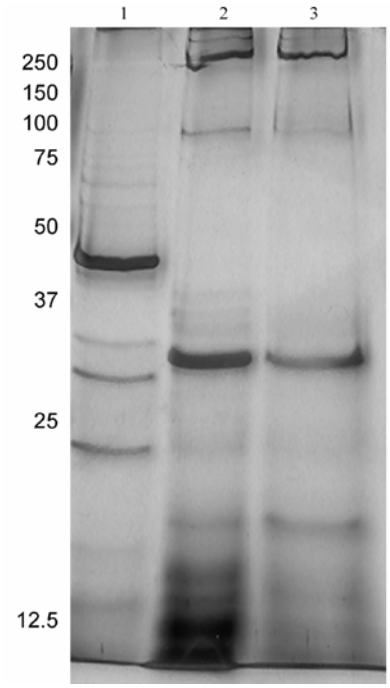


Figure 5.2 Outer membrane proteins profiles of *P. fluorescens* planktonic (lane 1) and cells within biofilms formed under turbulent (lane 2) and laminar (lane 3) flow regimes. Numbers on the left represent molecular weights in kDa.

According to Figure 5.2, the OMP profiles obtained with cells within biofilms (lane 2 and 3) differ considerably from the ones obtained with their free cell counterparts (lane 1). Moreover, different flow regimes (laminar and turbulent) applied for biofilm formation resulted in comparable protein expression. Nevertheless, the outer membrane of cells present in biofilms formed under turbulent flow regime (lane 2) exhibit a higher number of protein bands as well as bands with a higher intensity than the OMP present in cells within biofilms formed under laminar flow regime. It can be also observed that protein expression of cells within biofilms resulted in an increase of proteins bands with lower molecular weights.

The OMP has a significant role in a context of biofilm eradication by chemical treatment, since the bacterial outer membranes form an adaptative barrier to the external environment, protecting the bacterial cell contents from damaging substances (biocides and other antimicrobial agents) when disinfection procedures are applied - while allowing the selective uptake of nutrients (Nikaido, 1996). This study revealed that the OMP profiles obtained with cells within biofilms (Figure 5.2) differed greatly from the OMP profiles of planktonic cells. These results are in accordance with the approaches made by several authors (Costerton *et al.,* 1995b; Coquet *et al.,* 2002, Sauer *et al.,* 2002; Wang *et al.,* 2003), proposing that bacterial adhesion to a surface triggers the expression of a number of genes, making the biofilm cells clearly phenotypically different from their free cell counterparts. However, that are no previous reports concerning the influence of the flow regime in the biofilm phenotype.

Since the phenotypic characteristics of cells in planktonic and in sessile state, even cells from biofilms formed under different flow regimes, differed significantly, a PCR procedure was followed in order to ascertain the genetic stability of cells in different states.

Figure 5.3 presents the PCR results of DNA from planktonic cells and from cells within biofilms formed under different flow regimes.

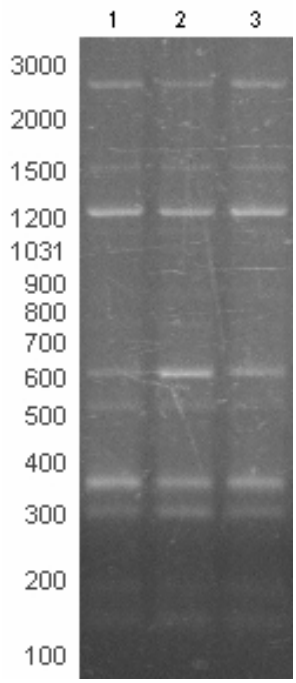


Figure 5.3 DNA profiles of *P. fluorescens* planktonic cells (lane 1) and of cells within biofilms formed under turbulent (lane 2) and laminar flow regime (lane 3).

As it can be confirmed in Figure 5.3, the DNA profiles are similar for the three conditions studied, meaning that no genotypic differences existed, thus it can be said that no contamination occurred during biofilm formation, or that sessile cells were genotipically similar to the planktonic ones.

The phenotypic differences within cellular states have significant impact on the design of biofilm control procedures since current disinfection procedures are based on tests using cells in planktonic state (EN 1276, 1997; Wirtanen *et al.,* 2001). One of the earliest observations of such altered behaviour is the increased resistance of biofilm cells to antimicrobial agents when comparing planktonic and biofilm states (Wirtanen *et al.,* 2001). This study reveals basic phenotypic characteristics of *P. fluorescens* in different cellular arrangements, linking with their behaviour in a disinfection process. Besides the existence of a well known EPS matrix involving the cells within the biofilms, new factors can be behind the recalcitrance of biofilms as the reduced cellular size and high cellular number; low metabolic activity; distinct biochemical composition of cells within biofilms from the ones in planktonic state; changed OMP and expression of a larger number of proteins. These features will be of crucial importance in a context of biofilm control. Drenkard and Ausubel (2002), studying *P. aeruginosa* biofilms, also speculated that resistant phenotypic variants were responsible for the increased resistance to antimicrobial agents. According to the same

authors, *P. aeruginosa* is capable of undergoing transient phenotypic changes that allow the bacteria to increase their antibiotic resistance both *in vitro* and *in vivo*. Several authors (Costerton *et al.,* 1995b; Davies and Geesey, 1995; Watnick and Kolter, 2000; Sauer and Camper, 2001; Sauer *et al.,* 2002) also suggested that biofilms express others specific survival mechanisms – expression of specific resistance genes; decreased growth rate; restricted penetration; biofilm-specific substances such as polysaccharides; quorum sensing specific effects –that explain their remarkable resistance to antimicrobial agents. In previous studies related with the use of biocides to control *P. fluorescens* growth, it was found considerable resistance to the antimicrobials when the cells were entrapped in a biofilm (Pereira and Vieira, 2001). From the present work it can be ascertained that bacteria express a phenotype mutant when face changes in the environmental and hydrodynamic conditions that can account to these differences.

5.3.2 Comparison of biofilms formed under different hydrodynamic conditions

A better understanding of biofilm structure and behaviour is essential for the establishment of specific and reliable methods to control biofilms, since biofilms are increasingly recognized as highly organized and dynamic habitats (Reisner *et al.* 2003). In this work, biofilms were formed in a continuous flow cell reactor. This type of device offers a simple and effective possibility to study and characterize biofilms in a well-controlled and reproducible manner (Pereira *et al.,* 2002a). Table 5.2 presents the biofilm mass and the amount of proteins and polysaccharides presented in turbulent and laminar biofilms.

Table 5.2 Characteristics of *P. fluorescens* turbulent and laminar biofilms in terms of biofilm mass, proteins and polysaccharides composition. Mean ± SD

		Biofilm	
		Turbulent	**Laminar**
Biofilm mass (mg/cm^2)		1.71 ± 0.23	0.717 ± 0.19
Proteins (mg/g biofilm)	**Total**	255 ± 77	71.3 ± 25
	Matrix	37.4 ± 6.7	19.8 ± 7.0
	Cells	218 ± 83	51.5 ± 18
Polysaccharides (mg/g biofilm)	**Total**	136 ± 33	239 ± 84
	Matrix	115 ± 28	167 ± 59
	Cells	21.4 ± 5.2	71.5 ± 25

The characterization of *P. fluorescens* biofilms showed that turbulent biofilms had about two times more mass *per* cm^2 than the laminar ones. Concerning the amount of proteins, biofilms formed under turbulent flow resulted in a higher content of total proteins. The protein content differed in turbulent and laminar biofilms since there was a considerably higher amount of proteins in turbulent biofilms cells. The proteins present in the matrix were in an equal extent in the different flow regime biofilms studied. The polysaccharide content was in a higher amount in laminar biofilms for every condition analyzed, being this biofilm constituent in a higher extent in the biofilm matrix than in the cellular composition.

Figure 5.4 presents photographs of stainless steel coupons with surfaces covered with biofilms developed under different flow regimes.

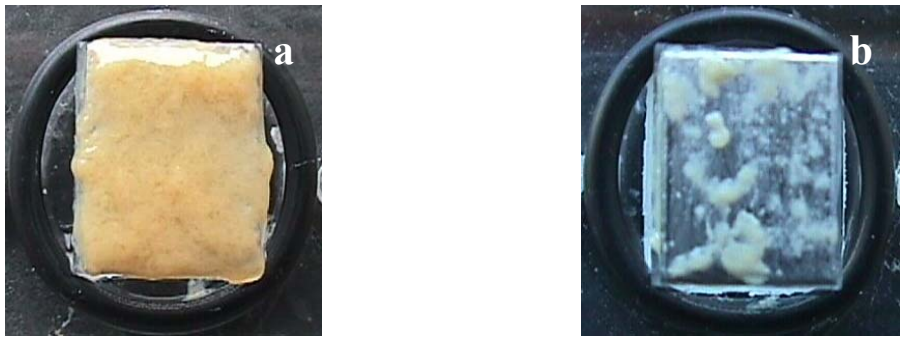


Figure 5.4 Photograph of the coupons with biofilms formed on the stainless steel slides under turbulent (**a**) and laminar flow (**b**).

The results obtained showed that biofilm structure depends on the flow conditions since turbulent biofilms appeared homogeneous and slimy while laminar biofilms were scattered on the surface.

Figure 5.5 displays microphotographs representative of the several fields observed in each biofilm-covered metal surface.

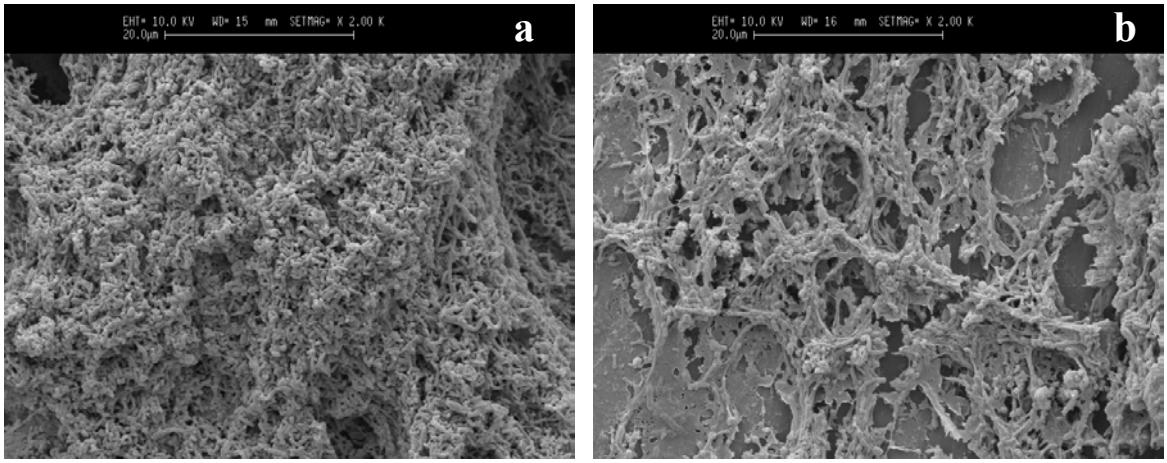


Figure 5.5 Scanning electron microscopy photomicrographs of a 7 d old *P. fluorescens* biofilm formed on stainless steel slides under turbulent (**a**) and laminar (**b**) flow. X 2000 magnification, bar=20 μm.

As observed in Figure 5.5, biofilms grown under turbulent flow look very different from the ones grown under laminar flow. Therefore, the hydrodynamic conditions play an important role in the biofilm architecture being responsible for the differences obtained. It is also possible to observe that biofilms formed in the stainless steel surfaces under laminar flow do not cover totally the surface, corroborating the result obtained in Figure 5.4. Furthermore, it is evident the existence of a greater amount of cells in the biofilms formed under turbulent flow as well as an almost inexistent biofilm matrix when compared with the biofilms formed under laminar flow. According to Fux *et al.* (2005a), mature biofilms demonstrate a complex 3-dimensional structure with numerous microenvironments differing with respect to osmolarity, nutritional supply and cell density, being the biofilm architecture a response to a stochastic process (Davey and O`Toole, 2000). The heterogeneity found within biofilms could be behind the reduced susceptibility of sessile microorganisms face to antimicrobial agents, which could lead to the existence of persistent microorganisms that are recalcitrant to further treatments (Stewart, 2003).

Biofilm formation occurs as a result of a sequence of events: microbial surface attachment, cell proliferation, EPS matrix production and detachment. This process is partially controlled by quorum sensing, an inter-bacterial communication mechanism that is dependent on population density and is associated with radical changes in protein expression (Sauer *et al.,* 2001). However, in this work, it seems that quorum sensing phenomenon does not interfere with the biofilm characteristics. Since it is a flowing system in which the diluted medium is continually refreshed and is from the same provenience for both biofilms formed under turbulent and laminar flow, it is possible that the diffusible signal molecules to be washed out of the biofilm as proposed by Purevdorj *et al.* (2002), being the cell signalling mechanisms mollified when comparing both biofilms in the present study. Additionally, according to Purevdorj *et al.* (2002), quorum sensing alone is not necessarily required for biofilm formation and other factors of the growth environment, including hydrodynamic conditions can play a role of greater significance in determining the biofilm structure. Liu and Tay (2001) also stated that physical phenomenon rather than biological effect is responsible for the observed relation between hydrodynamic shear and biofilm structure.

Previous studies made by Pereira *et al.* (2002b) revealed that turbulent biofilms are thicker than laminar biofilms. Since thickness can give rise to increased diffusional limitations, that biofilm property have, thus, obvious influence on the microbial metabolism of the entrapped cells. Nevertheless, cells within turbulent biofilms are more actives, being, probably, the glucose consumption due to the higher number of cell *per* g of biofilm, which determined differences in the biochemical characteristics. Also, the fact that under turbulent flow the velocity is higher than under laminar flow resulted in a much higher rate of substrate transport from the fluid to the biofilm surface, which, probably, also favoured the formation of a more active biofilm.

This study shows that the idea that under submission to stronger hydrodynamic conditions the cells build their habitat by producing more EPS does not happen under the tested conditions (Table 5.2). In fact, it has been currently observed (Lazarova *et al.,* 1994; Liu and Tay, 2001; Pereira *et al.,* 2001) that high detachment forces can induce the biofilms to secrete more EPS, which in turn would result in a balanced biofilm structure under the given hydrodynamic conditions. With the experimental conditions followed in this study, the higher flow velocity contributed to the adhesion of higher number of cells rather than the production of an extensive EPS matrix.

5.4 Conclusions

Profound modifications induced by the immobilized status of bacteria within biofilms have been highlighted in this chapter. This particular physiology of cells within biofilms may help to explain their extraordinary phenotypic properties when compared with suspended bacteria. Planktonic cells look and behave significantly different from sessile cells. Planktonic cells presented a more elongated shape, were more active, and had a higher content of proteins and polysaccharides *per* cell than the cells present in biofilms. Nevertheless, the number of cells *per* mg of biological mass was much higher in biofilms. The OMP profiles of cell within biofilms were considerably different from their free cell counterparts. Nevertheless, the effect of different flow regimes (laminar and turbulent) in biofilm formation resulted in similar OMP profiles. Concerning biofilm formation under different flow regimes, the turbulent biofilms revealed to be more active and had a higher number of cells than the laminar ones. The cellular proteins and polysaccharides were in a higher extent in cells within laminar biofilms. Concerning the comparison of turbulent and laminar biofilms, the ones formed under turbulent flow had about two times more mass *per* cm^2, had a higher content of total proteins due to the higher amount of cellular proteins. Laminar biofilms had a higher content of cellular and extracellular polysaccharides than turbulent biofilms, being this biofilm constituent in a higher extent in the extracellular composition. Furthermore, direct visualization and SEM photomicrographs show that biofilms formed under different flow regimes also look very differently.

This ability of biofilms to adapt their morphology to different environmental conditions may help to explain their tenacious nature and recalcitrant to control and his more detailed understanding of the complex roles of environmental factors in the biofilm behaviour will lead to improve the strategies for biofilm control.

Chapter 6

Inactivation of planktonic *Pseudomonas fluorescens* using aldehyde-based biocides and surfactants – studies of the mechanism of action of the antimicrobial agent

Abstract

This preliminary study is made in order to determine the antimicrobial efficacy of aldehyde-based biocides (GTA, OPA) and the surfactants (CTAB and SDS). It is also a goal to obtain experimental data that can help to deep understand the mode of action of these antimicrobial agents. This is made by assessing the bacterial respiratory activity, as a measurement of the bacteria viability, and the ATP released. Assays in the presence of BSA are also carried out in order to simulate a disinfection process under dirty conditions. Phenotypic changes (OMP and cellular colour changes) are carried out and compared with the bacterial phenotype before chemical treatment.

6.1 Introduction

Control of microbial growth is required in food plant operations where wet surfaces provide favourable conditions for proliferation of microorganisms. The aim of the disinfection process is to reduce the surface population of viable microorganisms after cleaning and to prevent microbial growth on surfaces before restart of production (Gibson *et al.,* 1999; Wirtanen *et al.,* 2001).

Determination of disinfectant efficiency is often performed in suspension tests with ready-to-use dilutions. The European Committee for Standardization (CEN) has launched many standards. The microorganisms used are standard test microorganisms as well as spoilage bacteria, pathogens and spores of concern in hygiene. All disinfectants passing the efficacy test should reduce the number of vegetative cells by ≥ 5 log units and the number of bacterial spores by ≥ 1 log unit (Wirtanen *et al.,* 2001). One of the several existent tests in European Standards is the quantitative suspension test EN 1276:1997, where the bactericidal activity of a disinfectant for use in food, industrial, domestic and institutional areas is tested against both Gram-negative (*Pseudomonas aeruginosa* and *Escherichia coli*) and Gram-positive (*Staphylococcus aureus* and *Enterococcus hirae*) bacteria in hard water. Additionally, bacteria as *Salmonella typhimurium*, *Lactobacillus brevis* and *Enterobacter cloacae* can be used if needed. Bovine serum albumine can also be included in the test solutions as an organic soil at two concentrations 0.03 % (to mimic clean conditions) and 0.3 % (for dirty conditions).

GTA and OPA are two aldehydes that are known to have good antimicrobial properties. OPA is a new product that is claimed to have excellent microbiocidal, mycobactericidal and sporicidal activity (McDonnell and Russell, 1999; Rutala and Weber, 2001). OPA received clearance by FDA (Food and Drug Administration) in October 1999 and is currently under study as an alternative to glutaraldehyde (GTA) for high level disinfection. OPA is an aromatic compound with two aldehyde groups. The aromatic component might allow OPA to penetrate the outer layers of cells, thus helping to explain the very high activity of this biocide against Gram-negative bacteria even thought the degree of cross-linking seems to be less than with GTA (McDonnell and Russell, 1999; Walsh *et al*, 1999b). OPA has several potential advantages compared to GTA: is odourless, stable and effective over a wide pH range of 3-9 (Rutala and Weber, 2001), non-irritating to the eyes and nasal passages and does not require activation before its use.

Surfactants are commonly used in mixtures of cleaning products because of their ability of lowering surface and interfacial tensions of liquids. So, surfactants have the ability to wet surfaces, penetrate soil and solubilize fatty materials (Christofi and Ivshina, 2002; Glover *et al.,* 1999). QAC´s are often employed as disinfectants for manual processing lines and surfaces in the food industry, and in human medicine area (Mereghetti *et al.* 2000), because of their excellent hard-surface cleaning, deodorization and antimicrobial properties (McDonnell and Russell, 1999). QAC´s mode of action is attributed to their positive charge, which forms an electrostatic bond with negatively charged sites on microbial cell walls (McDonnell and Russell, 1999). Those electrostatic bonds create stress in the cell wall, leading to cell lysis and death. QAC´s also cause cell death by protein denaturation, disruption of cell-wall permeability and reduction of the normal intake of life-sustaining nutrients to the cell (Cloete *et al.* 1998). CTAB is a QAC that appears to rupture the cell membrane. The primary site of action of CTAB has been suggested to be the lipid components of the membrane causing cell lysis as secondary effect (Gilbert *et al.* 2002). Anionic surfactants possess strong detergent but weak antimicrobial properties, except at high concentrations, when they induce lyses of Gram-negative bacteria (Hugo and Russell, 1982). The outer and cytoplasmic membranes and the membrane-bound enzyme environment and function are the main targets of anionic surfactants (Denyer and Stewart, 1998). SDS is an anionic surfactant widely used in detergent formulations (Jèrábkova *et al.,* 1999).

The aim of this work is to clarify the antimicrobial potential of four chemical agents against *P. fluorescens* in planktonic state. To investigate the possible interference of proteins with the biocidal action of antimicrobial agent, further tests are performed with the presence of a model protein in order to mimic dirty conditions found in a real situation, according to the EN 1276 (1997).

6.2 Material and methods

6.2.1 Microorganism

Pseudomonas fluorescens ATCC 13525^{T} was used through this work.

6.2.2 Disinfection procedure

A suitable volume of the bacterial culture was harvested from the 2 l reactor, described in the sub-chapter 3.2.3, and washed with saline phosphate buffer by three consecutive steps of centrifugation (3777 *g*, 5 min) and resuspended in phosphate buffer pH 7 in order to obtain a suspension with an optical density (λ = 640 nm) of 0.4 (~ 1×10^{9} cells/ml). This bacterial culture was then divided by several sterilised glass flasks, put in an orbital shaker (120 min^{-1}, 27 ºC), and exposed to different concentrations of the various antimicrobial agents for ½ h.

The effect of the chemicals was assessed by determining the bacterial respiratory activity through oxygen consumption, the assessment of the ATP release and the analysis of the OMP expression (sub-chapter 3.6). Before the assessment of the bacterial respiratory activity, the antimicrobial agents were neutralized according to the procedure described in sub-chapter 3.5.1.

To investigate the influence of the proteins on the antimicrobial efficacy, the procedure described above was followed but with the previous addition of 3 g/l (European Standard –EN 1276) of BSA (Merck 12018) to the bacterial suspension, simulating, by this way, dirty conditions.

6.2.3 Antimicrobial agents

The follow chemical agents were used:

GTA at 200, 400, 800, 1500, 2000, 3500, 5000, 7000, 10000 and 13000 mg/l;

OPA at 2.5, 5, 15, 25, 35, 45, 55, 65, 100, 200 and 300 mg/l;

CTAB at 0.125, 0.250, 0.500 and 0.900 mM;

SDS at 0.5, 1, 3 and 7 mM.

6.2.4 Adenosine triphosphate measurement

In order to investigate possible interference of the proteins with the bioluminescent method, some control experiments were made with phosphate buffer in the presence and absence of BSA. This control was only made with the surfactant CTAB. The effect of the chemical agents on the bacterial integrity was also evaluated in terms of relative light units as an estimative of the intracellular ATP content released. The latter was calculated, according to Dalzell and Christofi (2002), using the equation below:

$$Relative\ light\ units = (RLU_1/RLU_0) \quad (6.1)$$

where RLU_0 is the relative light units of the control assay (bacteria without chemical addition) and RLU_1 is the relative light units of the test sample.

6.2.5 Cell growth in the presence of sodium dodecyl sulfate

To determine whether the presence of SDS had any effect on the ability of the bacteria to grow, several sterile flasks were prepared with 200 ml of sterile growth medium, inoculated with an overnight bacterial suspension in a volume enough to have an $OD_{(640\ nm)}$ 0.200 ($\sim 5 \times 10^8$ cells/ml). The several bacterial cultures were incubated in an orbital shaker (120 min^{-1}, 27 ºC). To each one of the flasks a different SDS concentration (0.5, 1, 3 and 7 mM) was added. A control test, without surfactant addition, was also performed. The bacterial growth was followed temporarily by taking aseptically a 2.5 ml sample from each flask and recorded its OD.

In order to examine if the bacteria could use SDS as an additional carbon source, the procedure described above was followed, with the difference that the growth medium was prepared without glucose.

At the end of the overall experiments (that were performed in triplicate for each situation studied), the cultures were streaked onto solid medium to ensure that the cultures remained uncontaminated.

6.2.6 Respiratory activity

The bacterial respiratory activity assays were performed in a Yellow Springs BOM (Model 53) according to the procedure described in sub-chapter 3.6.6.

6.2.7 Mass of bacteria

The mass of bacteria present in each glass flask was estimated by the determination of the TVS of the bacterial cultures (sub-chapter 3.6.5).

6.2.8 Outer membrane proteins analysis

The OMP profiles of cells without treatment (control) and after treatment for ½ h with 200 mg/l of GTA, 100 mg/l OPA, 0.9 mM CTAB and 7 mM of SDS were analyzed according to the procedures referred in sub-chapter 3.6.8.

The colour of each bacterial pellet, after chemical treatment, was recorded and compared with the starting colour of the microorganism without chemical treatment.

6.2.9 Statistical analysis

The Student's *t* test was performed when the aim was to investigate whether the differences between the experimental values obtained under different conditions could be considered significant.

6.3 Results and discussion

6.3.1 Action of ortho-phthalaldehyde against Pseudomonas fluorescens planktonic cells

The effect of OPA on the respiratory activity of *P. fluorescens* in the presence and absence of BSA is presented in Figure 6.1.

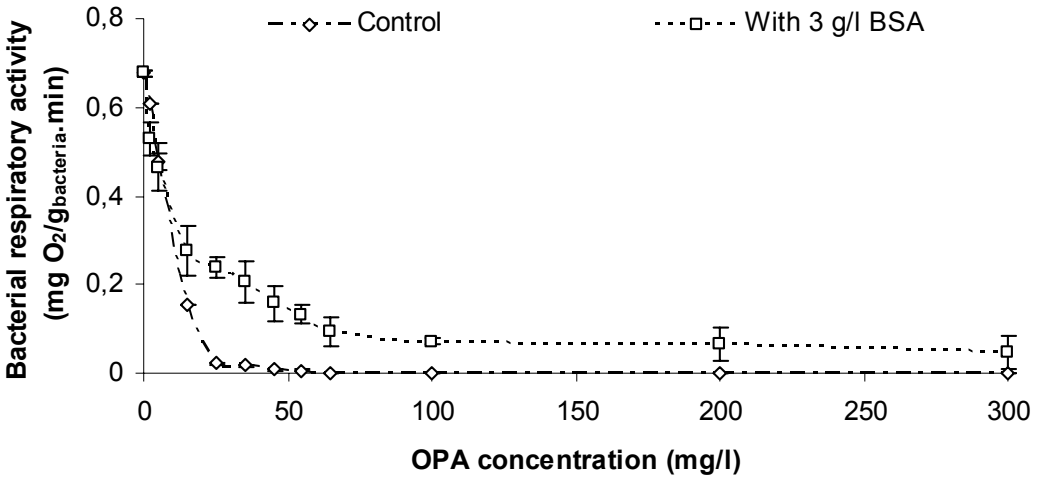


Figure 6.1 Respiratory activity of the bacterial suspended cultures, after treatment with several concentrations of OPA, with and without (control) BSA addition.

From Figure 6.1, it can be seen that an increase in the OPA concentration promoted a decrease in the bacterial activity. For concentrations equal and higher than 65 mg/l total bacterial inactivation was achieved. This bactericidal effect of OPA is attributed to its lipophilic aromatic nature that makes it easy to uptake through the outer layers of Gram-negative bacteria (Simons *et al.*, 2000). However, the antimicrobial action of OPA was severely affected in the presence of BSA. In fact, the antimicrobial activity of OPA was significantly reduced when the BSA was added to the bacterial suspended cultures, being this reduction particularly noticeable for OPA concentrations higher than 15 mg/l. In the presence of BSA, total inactivation of the bacterial suspension was not observed even when high OPA concentrations were used. This phenomenon is a consequence of the high reactivity of OPA with proteins (Walsh *et al.,* 1999a; 1999b). Nevertheless, besides the possible reactivity of OPA with primary amine presented in the protein, it can be also neutralized by the glycine of the BSA. In fact, it is known that BSA contains lysine, hystidine and glycine and that glycine can be used to neutralize the OPA and make it safe

for disposal (Rutala and Weber, 2001). Statistical analysis of the results obtained in the presence and absence of BSA showed that they were significantly different ($P < 0.01$).

A bioluminescence assay to determine ATP release was made as an attempt to ascertain if the antimicrobial action of OPA could have effect on the bacterial integrity (Figure 6.2).

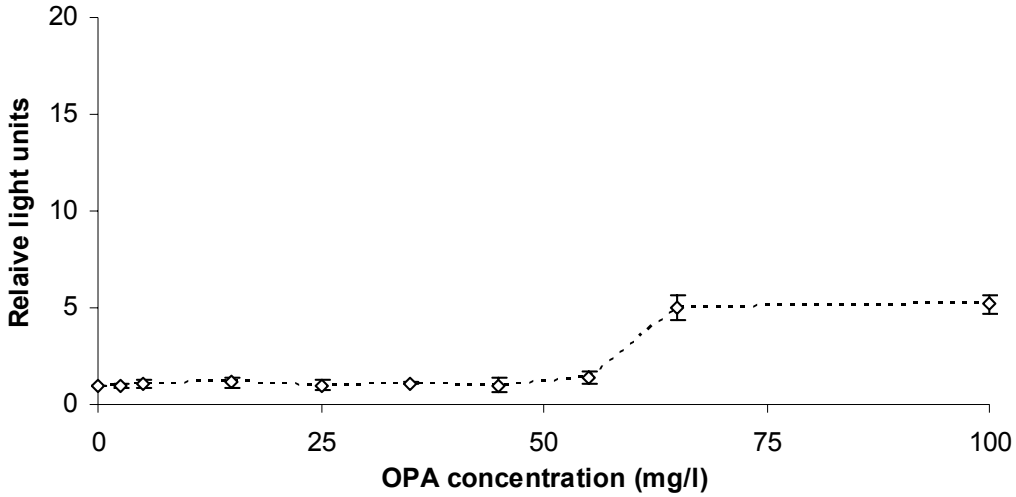


Figure 6.2 Relative light units as a measure of the ATP released from the bacterial cells after treatment with several concentrations of OPA.

Figure 6.2 shows that cellular products were not released when the cells were exposed to the biocide since the ATP content of the bacterial cultures, after OPA treatment, is almost insignificant and similar to the one observed in the control conditions. A six relative light units can not be considered significant and, thus indicative of the maintenance of the bacterial integrity.

6.3.2 Action of glutaraldehyde against Pseudomonas fluorescens planktonic cells

Figure 6.3 presents the bacterial respiratory activity after submitting the cells, in the presence and absence of BSA, to treatments with several concentrations of GTA.

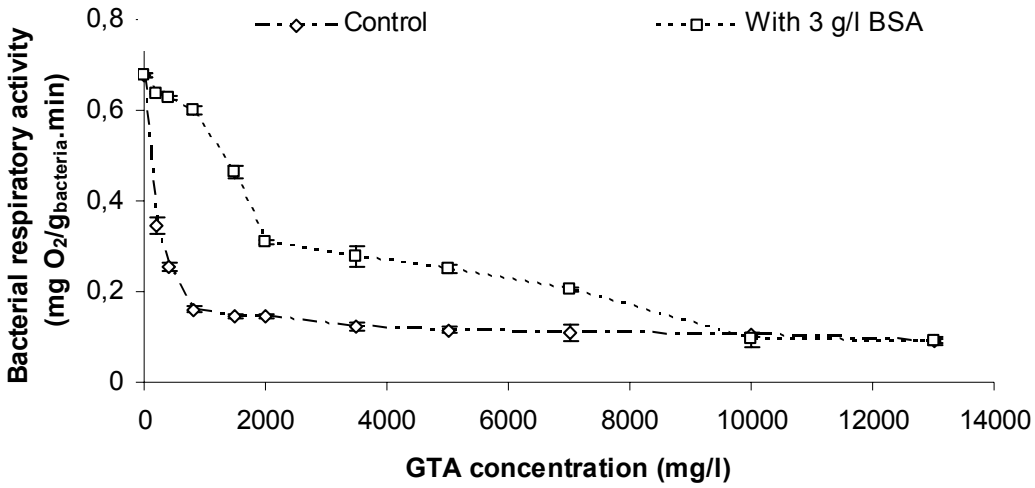


Figure 6.3 Respiratory activity of the bacterial suspended cultures, after treatment with several concentrations of GTA, with and without (control) BSA addition.

The results of bacterial respiratory activity after GTA application show that this biocide was not effective in the total inactivation of the cells. Even after the treatment with 13000 mg/l of GTA the cells still to have respiratory activity. Nevertheless, an increase in the GTA concentration promoted a decrease in the bacterial respiratory activity. Concerning the interference of BSA with the antimicrobial action of GTA, a statistical analysis reveals that the results without and with BSA presence are completely different ($P < 0.05$). In fact, in the presence of BSA, the reduction in the bacterial respiratory activity caused by GTA was drastically diminished. This reduction might be explained through the reaction of the aldehyde group with amines since BSA is a protein that contains primary amine residues. Moreover, histidine also enters in the constitution of BSA, being this amino acid suggested as a neutraliser for aldehyde biocides in the European Standard Test (Walsh *et al*., 1999b). Consequently, BSA reduces the availability of the groups necessary for biocidal activity. Moreover, BSA also decreases the accessibility of the biocide to its target sites by forming a coating around the microbial cell (Fraud *et al*, 2001).

Figure 6.4 shows the relative light units as an estimative of the ATP possible released by the cells due to the exposure to several concentrations of GTA.

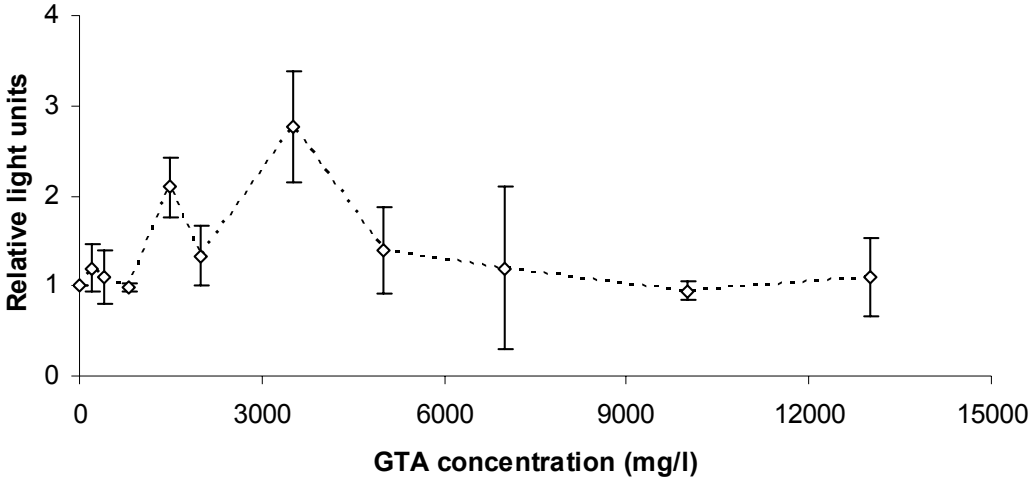


Figure 6.4 Relative light units as a measure of the ATP released from the bacterial cells after treatment with several concentrations of GTA.

The ATP bioluminescence assay showed that the RLU detected in the bacterial suspensions after GTA treatment are insignificant and similar to the RLU observed in the control assay (without GTA addition). So, it is possible to say that cellular disruption did not occur when the cells were exposed to this biocide, as already observed with OPA. GTA was already demonstrated to be a protective agent against cell lysis (Azeredo *et al.,* 2003a).

6.3.3 Comparison of the antimicrobial effects of ortho-phthalaldehyde and glutaraldehyde

Comparing results of GTA and OPA, it is clear that OPA is more effective in the inactivation of suspended cells than GTA. In fact, the concentration of OPA needed to complete inactivate the suspended bacterial cultures was about 100 mg/l, whereas for GTA even with a concentration of 13000 mg/l there was no total inactivation. This latter evidence emphasizes OPA as a possible alternative to GTA for high level disinfection, as already suggested by Walsh *et al.* (1999a). The mechanism of antimicrobial action of GTA and OPA may be related with the characteristics of the chemicals, since GTA and OPA are known to stabilize the outer membrane and cell walls of vegetative bacteria (Walsh *et al.,* 1999b) acting, probably, by blocking the passage of essential products to the cell.

The OPA and GTA efficacy was considerably reduced when BSA was introduced in the suspended bacterial cultures. The reasons for that reduction are probably related with the properties of BSA, since it can act as neutralizer of GTA and OPA (Walsh *et al.*, 1999a; 1999b; Rutala and Weber, 2001), lowering, thus, the amount of biocide available to react

with the cells. The possible formation of a BSA layer coating the cells and, thus, impairing the access of biocides to the bacteria, can also account for the reduction of the bactericidal efficacy of both biocides when the protein was presented in the cultures

6.3.4 Action of cetyltrimethyl ammonium bromide against Pseudomonas fluorescens planktonic cells

The effect of CTAB on the bacterial cells is presented in Figures 6.5 and 6.6.

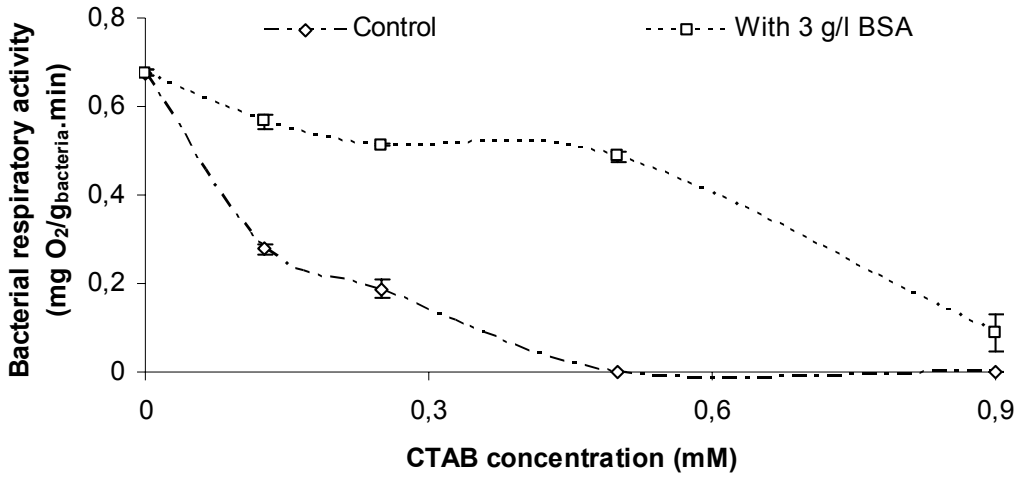


Figure 6.5 Respiratory activity of the bacterial suspended cultures, after treatment with several concentrations of CTAB, with and without (control) BSA addition.

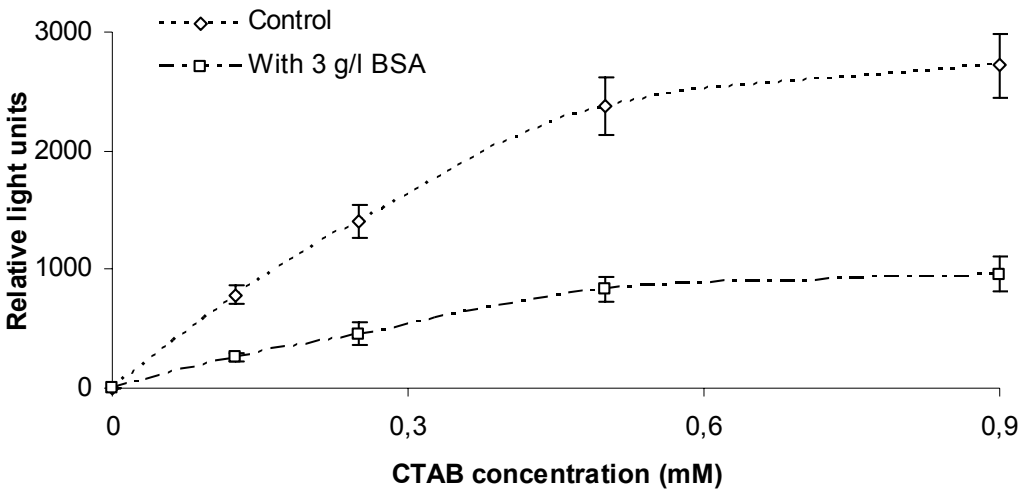


Figure 6.6 Relative light units as a measure of the ATP released from the bacterial cells after treatment with several concentrations of CTAB, with and without (control) BSA addition.

Figure 6.5 shows that the respiratory activity decreases with the CTAB application, being this decrease function of the CTAB concentration increase. The total bacterial inactivation was achieved for concentrations higher than 0.5 mM. However, once more, the presence of BSA reduced significantly ($P < 0.05$) the antibacterial efficacy of CTAB. The ATP released into the medium, after CTAB aggression, increased as the CTAB concentration increased (Figure 6.6), suggesting that this surfactant promotes cellular disruption in an extent dependent of the CTAB doses. This result was not surprising, since QAC´s are believed to damage the outer membrane of Gram-negative bacteria, thereby promoting the release of cellular constituents (McDonnell and Russell, 1999). Since CTAB promoted ATP release, an additional ATP bioluminescence experiment performed, evaluating, in this time, the ATP released by the cells when treated with CTAB, but in the presence of BSA. Figure 6.6 shows that the RLU observed in the experiments made in the presence of BSA are always inferior when compared without BSA presence, regardless the CTAB concentration applied. This observation is a clearly sign of the BSA ability to protect the bacterial cells. This fact helps to understand why, even with CTAB concentrations higher than 0.5 mM, total bacterial inactivation was not achieved. So, the presence of BSA has a protective effect against CTAB action, leading to the release of a lower ATP concentration ($P < 0.05$).

Comparing the results obtained by respirometry and the bioluminescent methods, a strong relationship ($R^2 = 0.975$) was found between bacterial inactivation and relative light

units, meaning that an increase in bacterial inactivation corresponds to a proportional increase in the ATP released by the cells ($P > 0.1$). Concerning the same data but in the presence of BSA, the correlation coefficient found, $R^2 = 0.602$, was undoubtedly poor. This latter relationship may indicate that when BSA was present in suspension, bacterial inactivation was related to the ATP released and probably, the presence of BSA acted as a protective agent to the cells avoiding their disruption, as presented in Figure 6.6. According to Ishikawa *et al.* (2002), surfactants may disturb membrane structure through interaction with cellular components, in particular proteins and lipids, being, therefore, used to extract proteins from cell membranes (Chatterjee *et al.,* 2002). This fact can explain the diminished antibacterial effect of CTAB in the presence of proteins under dirty conditions (Figure 6.5), since part of the surfactant reacted with the BSA, and thus the amount of CTAB available for reaction with the cells was smaller.

6.3.5 Action of sodium dodecyl sulfate against Pseudomonas fluorescens planktonic cells

The results of the antimicrobial activity of SDS against planktonic cells of *P. fluorescens* are presented in Figures 6.7 and 6.8.

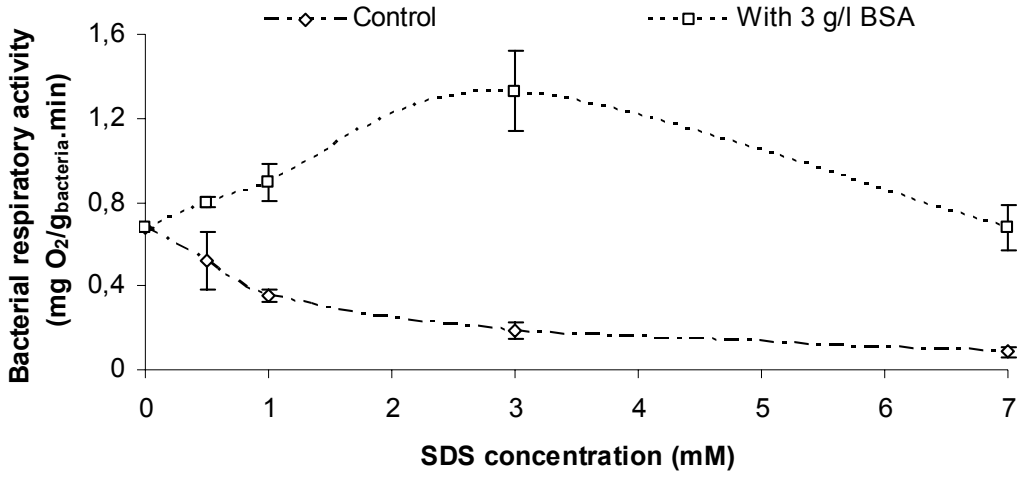


Figure 6.7 Respiratory activity of the bacterial suspended cultures, after treatment with several concentrations of SDS, with and without (control) BSA addition.

Figure 6.7 shows that SDS promotes significant reduction on the bacterial respiratory activity. Moreover, Figure 6.8 demonstrates that SDS did not promote the cellular disruption since the ATP released was similar to the value obtained in the control assay (without SDS addition). Therefore, it can be concluded that in the studied

experimental conditions, and in the range of concentrations tested, SDS is effective in the total inhibition of *P. fluorescens*.

Figure 6.7 also shows that, in the presence of BSA the bacterial respiratory activity increased reaching values even higher than the observed in the control. This fact suggests that the antimicrobial effect of SDS was neutralised and the bacterial activity stimulated. It seems that there was an optimal SDS/BSA rate that promoted surfactant neutralization and cellular activity induction. For instance, with the application of 3 mM of SDS and in the presence of BSA, the bacterial respiratory activity is approximately two times higher than the one obtained without SDS application. The statistical analysis of the results, obtained in the presence and absence of BSA, shows that they are significantly different ($P < 0.05$). The presence of BSA leads to the chemical neutralization of SDS. It is known that hydrophobic interactions are behind the reaction of surfactants with BSA in a controlled chemical system (Nelson, 1971; Gelamo *et al.,* 2002).

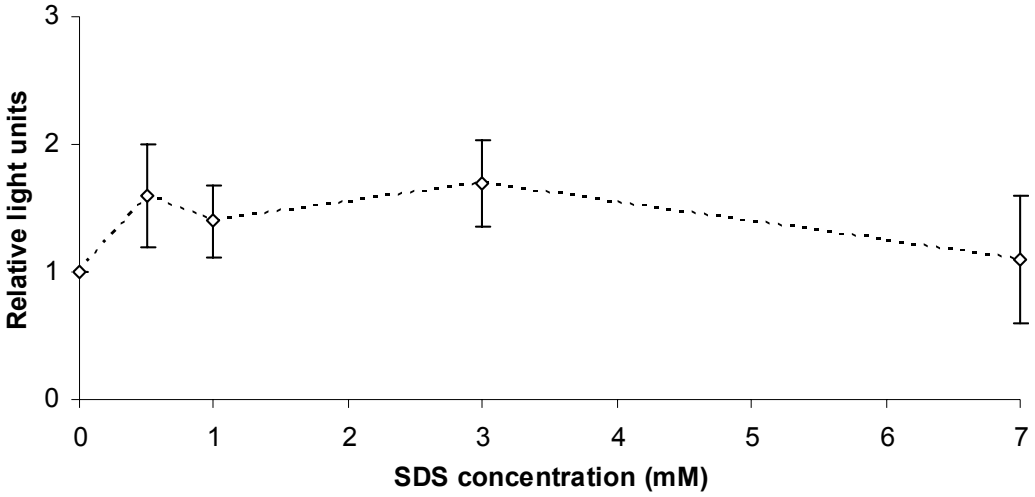


Figure 6.8 Relative light units as a measure of the ATP released from the bacterial cells after treatment with several concentrations of SDS.

The capability of a *Pseudomonas* spp. strain in metabolize SDS is a phenomenon previously described (Thomas and White, 1989; Singh *et al.,* 1998). So, in order to ascertain this capability with the strain used in this study, the bacterial growth was evaluated along time using SDS in two distinct approaches – as an additional (possible) growth source (Figure 6.9) and as an alternative to glucose (Figure 6.10).

Figure 6.9 shows growth *P. fluorescens* curves obtained in the absence (control) and presence of several concentrations of SDS.

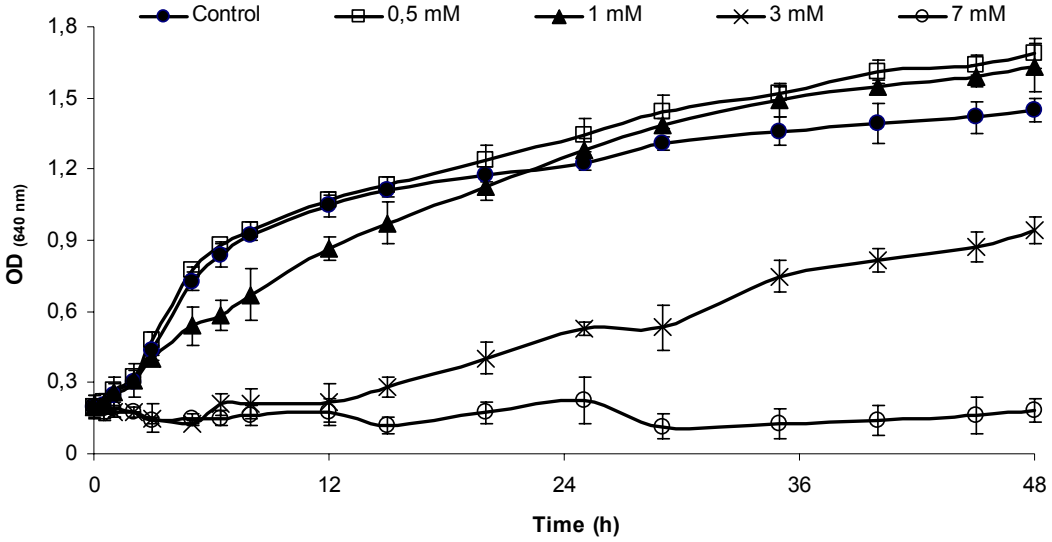


Figure 6.9 Growth curves of *P. fluorescens* with glucose as carbon source, in the presence and absence of SDS.

From the analysis of the cellular growth profiles, it can be seen that the OD of the bacterial cultures for the minor concentrations of SDS (0.5 and 1 mM), reached values higher than in the absence of SDS specially after 24 h of growth. Eventhough the growth curves in the presence of 0.5 and 1 mM of SDS had similar profiles with the one of the control curve, they are statistically different ($P < 0.05$). The presence of 3 mM of SDS caused a retarded cellular growth and reduced the final OD of the culture compared to growth curve with SDS absence ($P < 0.01$). The higher concentration of SDS (7 mM) promoted complete inhibition of growth. This inhibition of growth due to presence of the higher SDS concentration showed that this surfactant was not used as metabolized nutrient and that higher SDS concentrations were toxic to the cells. Additionally, diauxic growth was not found during the 48 h of experiment, for any situation tested.

In order to verify the possibility of *P. fluorescens* to use SDS as an alternative to glucose as a carbon source, some experiments were made in the absence of glucose in the growth medium.

Figure 6.10 shows the growth curves of *P. fluorescens* in the absence of glucose on its conventional medium and in the absence (control) and presence of several concentrations of SDS.

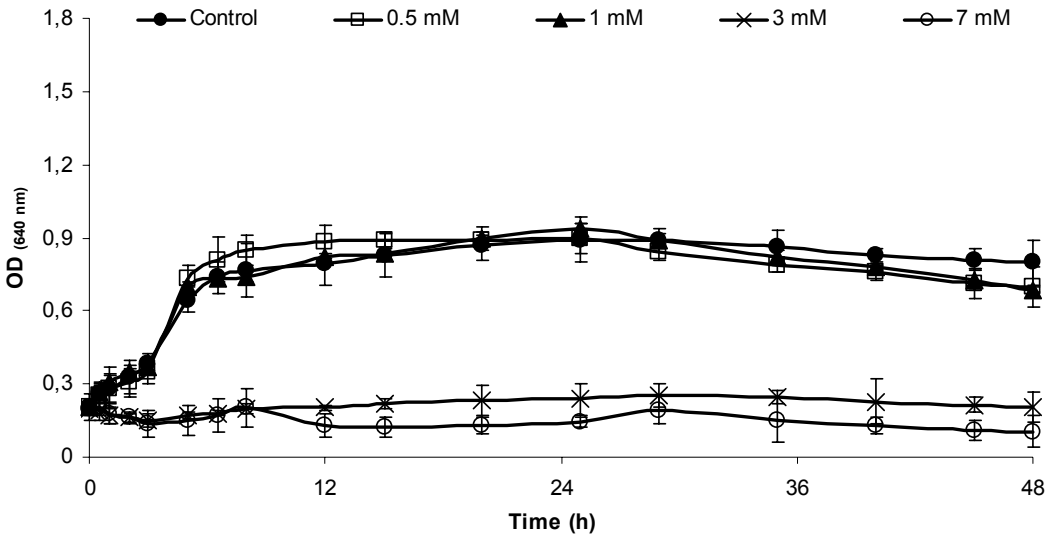


Figure 6.10 Growth curves of *P. fluorescens* without glucose, in the presence and absence of SDS.

From Figure 6.10, it can be seen that *P. fluorescens* grows in the absence of glucose as carbon source. However, SDS is not used as an alternative carbon source. Possibly, the cells found carbon sources in peptone and yeast extract, presented in the conventional medium. The growth patterns are similar without surfactant presence and in the presence of 0.5 and 1 mM ($P > 0.1$), proposing that *P. fluorescens* grows in conventional medium without glucose addition and that low concentrations of SDS are probably neutralized with the proteins presented in the growth medium. For the higher concentrations tested (3 and 7 mM), there was a toxic effect expressed in a decrease in the initial phase of the experiment, but the cells kept a latent state. The results obtained in the presence of 3 and 7 mM of SDS showed no statistical similarity with the control experiment ($P < 0.01$). The comparison between Figures 6.9 and 6.10, for the control assay, reinforces the phenomenon that SDS is not used as a carbon source. However, the growth extent is quite different in the presence and absence of glucose ($P < 0.01$). In the latter situation the cells reached the decline phase of growth more quickly (25 h after). This effect is also found in the presence of 0.5 and 1 mM of SDS, when comparing the experiments in the presence and absence of glucose the growth behaviour is different ($P < 0.01$). The test with 3 mM of SDS shows that in the absence of glucose the cells remain in a latent state of growth, whereas in the presence of glucose cellular growth was found ($P < 0.01$). In the presence of 7 mM of SDS, the cells had similar behaviour in the presence and absence of glucose ($P > 0.1$).

Thomas and White (1989) reported that *Pseudomonas* spp. could biodegrade SDS and incorporate the hydrophobic metabolites of the alkyl chain as cellular components, such as membrane lipids. Nevertheless, SDS seems not to be used as a substrate by the *P. fluorescens* used in this work.

Some possible mechanisms could explain the effects verified in the experiments in the presence of SDS: the surfactant is neutralized by the components present in the medium; SDS may inhibit growth; the interactions between the cells and the substrate may be altered in the presence of the surfactant, which can be due to an effect on the membrane permeability (Glover *et al.,* 1999) that was not enough to promote the membrane rupture, as suggested by the amount of ATP released. This subsequent surfactant-mediated uptake pathway, possibly, enhanced in some way the substrate uptake and, consequently, the respiratory activity, as found with the tests with BSA, where the presence of BSA and SDS stimulated the bacterial respiratory activity.

6.3.6 Comparison of the antimicrobial effects of cetyltrimethyl ammonium bromide and sodium dodecyl sulfate

Concerning the comparison of both surfactants, the chemical nature (anionic and cationic) has influence on the antimicrobial properties of the surfactants. CTAB promoted total bacterial inactivation with the application of 0.5 mM, a concentration half of the CMC (1 mM) and the reactivity with BSA promoted the decrease in the bacterial inactivation. The increase in bacterial inactivation occurred with the increase in the CTAB concentration. This surfactant also promoted the release of intracellular ATP being this release in a small extent with the presence of BSA due to its protective effects to the bacterial cells. Cationic surfactants have a general antimicrobial action. This lead to the disruption of the cell membranes, consequently to the depolarization of the cytoplasmic membrane and the leakage of the cytosol components. So, ultrastructural changes may be induced by the action of the cationic surfactants, producing dramatic effects on the bacterial envelopes and causing lysis or massive leakage of cell components (Rodríguez *et al.,* 2003).

Concerning the application of SDS, even with the application of 7 mM, a concentration near the CMC (8.3 mM), total inactivation was not achieved. The reactivity of SDS with BSA increased the bacterial respiratory activity for the smaller concentrations tested (0.5 and 1 mM), an effect verified in the growth curves, where SDS at the smaller

concentrations did not affected the cellular growth. Paulus (1993), already pointed out that the antimicrobial effect of anionic surfactants is restricted mainly to Gram-positive bacteria and that their point of attack is apparently the microbial cell membrane.

6.3.7 Effect of the antimicrobial agents in the bacterial phenotype: outer membrane proteins expression and colour changes

The OMP profile of *P. fluorescens* were assessed after treatment with 200 mg/l of GTA, 100 mg/l of OPA, 0.9 mM of CTAB and 7 mM of SDS in order to assess the effect of the several antimicrobial agents on the OMP expression and are presented in Figure 6.11.

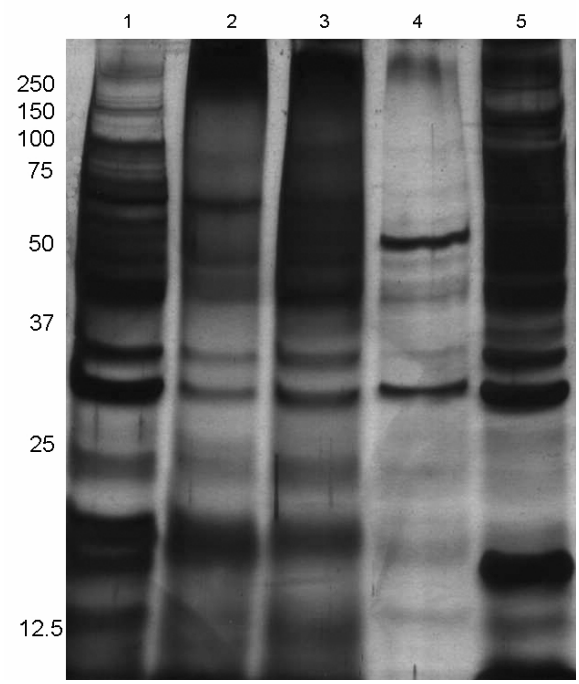


Figure 6.11 Outer membrane proteins profile of *P. fluorescens* cells without chemical treatment (lane 1) and after treatment with GTA (lane 2), OPA (lane 3), CTAB (lane 4) and SDS (lane 5). Numbers on the left represent molecular weights in kDa.

Comparing the OMP profiles without chemical treatment with the ones obtained from cells treated with the aldehyde-based biocides, no significant differences in the protein profiles are evident. As Walsh *et al.* (1999a) stated, the pre-treatment of cells with GTA and OPA inhibited cell lysis when challenged with other lytic agents. The treatment with the aldehydes caused a strengthening of the outer envelope, thereby protecting the cell from lysis. However, CTAB seems to promote a dilution in the OMP content presented in the electrophoresis gel after staining. Glover *et al.* (1999) reported that the action of cationic

surfactants cause damage to the cell membrane which results in the loss of its functions. This result corroborates, in some way, the result of ATP measurement because the existence of ATP release requires the destabilization of the outer membrane. It can be also observed that cells after treatment with SDS resulted in the increase of proteins bands with higher molecular weights. Several authors (Cloete *et al.,* 1998; Denyer and Stewart, 1999, Glover *et al.,* 1999; Ishikawa *et al.,* 2002) proposed that surfactants, due to their detergent properties, react strongly with cell outer membrane, particularly the membrane proteins.

Figure 6.12 shows the colour change in *P. fluorescens* after exposure to the several chemical agents during ½ h.

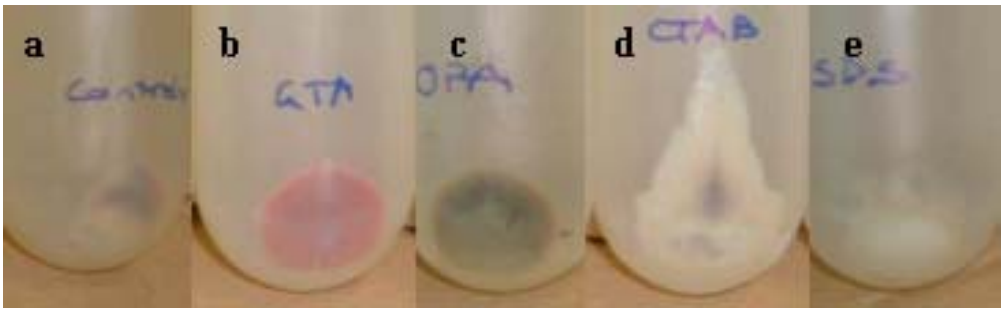


Figure 6.12 Colour of pellet of cells of *P. fluorescens* without chemical treatment (**a**) and after treatment with GTA (**b**), OPA (**c**), CTAB (**d**) and SDS (**e**).

From Figure 6.12, it is clear that the different chemical agents react with the bacterial cells promoting colour changes. Before exposure to the chemical agents, the pellet of cells look like pink, while after treatment with respectively 200 mg/l of GTA they look red/pink; 100 mg/l of OPA they look dark green; 0.9 mM of CTAB they look white/cream; 7 mM of SDS they look white.

The results of bacterial colour changes achieved after treatment with GTA and OPA are in accordance with the ones obtained by Walsh *et al.* (1999b), proposing that this effect is a consequence of the reaction with amino acids, consequently with proteins. According to Simons *et al.* (2000), the red colour developed after treatment with GTA resulted from an interaction of the dialdehyde with non-peptidoglycan components in the outer layers of Gram-negative bacteria. However, this reactivity of GTA and OPA with proteins does not affect the proteins of the outer membrane or affect in a small extent that is not detected in an SDS-PAGE analysis.

Concerning the bacterial colour changes after treatment with CTAB and SDS, there are no published reports concerning this effect. Nevertheless, it is not surprising that the

surfactants promoted the change in the bacterial colour, since, as stated above, the reactivity with the OMP and the detergent properties of surfactants promoted changes in the bacterial phenotype.

6.4 Conclusions

The results obtained with the bacteria in planktonic state will be used to foresee the effect of the chemical agents tested when applied to biofilms.

From this work several conclusions can be drawn:

The two aldehyde-based biocides (GTA and OPA) promoted reduction of the bacterial respiratory activity, being this effect more pronounced with the increase in the biocide concentration. Nevertheless, OPA was more effective in the inactivation of the bacterial cells than GTA.

Both CTAB and SDS promoted reduction in the bacterial activity. However, the surfactants reacted differently with bacterial cells, since CTAB promoted bacterial disruption and the consequent ATP release, while SDS did not have effect on the cell integrity.

The antimicrobial action of every chemical tested was significantly reduced when BSA was introduced in the suspended bacterial cultures. This fact proposes that the processes of disinfection under dirty conditions need the application of improved methods in order to avoid the effect of substances that react with the antimicrobial agents, lowering, by this way, the amount available to react with the cells. The presence of BSA, besides promoting the decrease in the antimicrobial efficacy of every chemical agent tested, acts as a cell protective agent when CTAB was applied. Also, it stimulated the bacterial respiratory activity when lower concentrations of SDS were applied.

The *P. fluorescens* strain used in this study had the capability to growth in SDS at low concentrations but concentrations of SDS near the CMC inhibited the bacterial growth.

The OMP of the bacterial cells is affected by the application of both surfactants. Even though aldehyde-based biocides have the ability to react with proteins, the OMP profile was not affected after treatment with GTA and OPA.

The several antimicrobial agents tested promoted bacterial colour changes, being this effect similar when comparing the effect found after the treatment with CTAB and SDS.

Chapter 7

Action of aldehyde-based biocides on the inactivation and removal of biofilms formed by *Pseudomonas fluorescens* under different flow regimes

Abstract

The effectiveness of GTA and OPA in the control biofilms formed by *P. fluorescens* on stainless steel slides, under turbulent and laminar conditions, using a flow cell reactor, is compared on this chapter. The action of the biocides is evaluated immediately and 3 h after treatment in terms of the activity of the biofilm and the mass of the biofilm that remained on the surface after the treatment. The experiment 3 h after treatment is an attempt to ascertain a prolonged effect on post-biocide application. Besides the experiment with two different biocides, the strategy of application of the biocide is also tested in order to assess whether time or concentration promotes a more efficient effect in the control of biofilms. The experimental tests with biofilms are performed with the application of a large range of concentrations of OPA for ½ h, concerning GTA, experimental tests are performed using a constant concentration and a range of exposure times.

7.1 Introduction

Antimicrobial agents have been the main weapons used to control unwanted biofilms, acting either by interfering with microbial metabolism or allowing the natural detachment from the surface (Chen and Stewart, 2000). GTA and OPA are two aldehydes that are known to have good antimicrobial properties. GTA is a dialdehyde that has a broad spectrum of activity against bacteria and their spores, fungi and viruses. The mechanism of action (Figure 7.1) involves a strong association with outer layers of bacterial cells, specifically with unprotonated amines on the cell surface (McDonnell and Russell, 1999). OPA is an aromatic compound with two aldehyde groups which action is believed to be due to the interactions with the primary amino groups of the outer envelope or cell wall. However, the level of cross-linking associated with the outer membrane does not appear to be as extensive as that of GTA (Walsh *et al.*, 1999a).

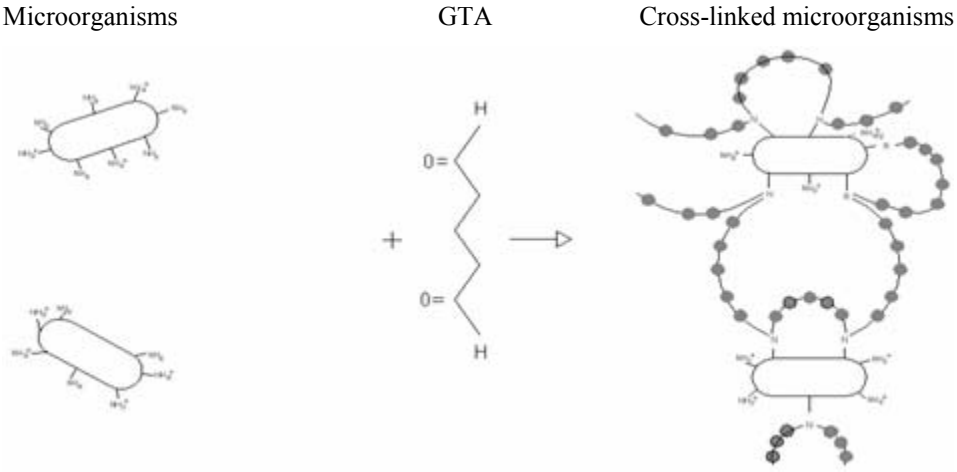


Figure 7.1 Mechanism of reaction between GTA and microorganisms (adapted from Eager and Leder, 1986).

GTA was already used for instance by Pereira and Vieira (2001) with the intent to control biofilms formed on stainless steel suspended plates, in laboratorial conditions. Concerning studies with OPA, this chemical is a promising antimicrobial agent (Alfa and Sitter, 1994; Walsh *et al.,* 1999a; 1999b; Rutala and Weber, 2001). Nevertheless, there is no information available concerning its application against biofilms.

The aim of this chapter is to evaluate the effect of OPA and GTA against biofilms formed under different flow regimes and to evaluate the effect of the mode of application on the biocide efficacy.

7.2 Materials and methods

7.2.1 Microorganism and culture conditions

The microorganism used was *P. fluorescens* ATCC 13525^{T}. The culture conditions were followed according to the procedure described in sub-chapter 3.1.2.

7.2.2 Biofilm system

The continuous flow cell system (sub-chapter 3.2) was used to perform biofilm formation under turbulent and laminar flow.

7.2.3 Biocides

OPA and GTA were tested throughout this work. In order to assess the best association of time of exposure and concentration, the biocides were applied against biofilms following two different approaches:

OPA was applied at a fixed time of exposure (½ h) but testing a range of concentrations (20, 50, 100, 200 and 300 mg/l);

GTA was used always at the same concentration (200 mg/l), but varying the time of contact (½ h, 1 h, 2 h, 2 times ½ h and 2 times 1 h). In the case of multiple applications they were spaced for 2 h.

Biocide solutions were diluted to the required concentration with sterile water.

7.2.4 Biofilm tests

After 7 d of growth, the biofilms formed on the metal slides of each parallel flow cell reactor, during 7 d, were exposed to OPA and GTA, according to the approaches described above. During the treatment period, the biocide solution replaced the diluted bacterial suspension flowing in the flow cell reactors. Each biocide concentration was tested in an independent experiment and each experiment was performed on three separate occasions. After the biocide exposure time, the flow of the biocide solution was interrupted and the bacterial suspension was re-introduced in the system in order to restore the conditions prior to biocide application and to mimic real situations encountered in industrial processes

(Pereira, 2001). In each experiment, after biofilm formation and prior to the initiation of the biocide treatment, three biofilm covered metal slides of each flow cell were sampled and used as control. The remaining biofilm-covered slides were sampled immediately after the exposure period to the biocide. The biofilms that covered the stainless steel slides were completely scraped and resuspended in phosphate buffer pH 7 and the residual biocide was neutralized by dilution (sub-chapter 3.5.1). Afterwards, the respiratory activity of the biofilms was assessed according to the procedure described in sub-chapter 3.6.6. After the assessment of the biofilm respiratory, the content of TVS of each biofilm suspension was quantified in order to determine the biofilm mass. To assess whether time plays a significant role on the action of the biocides, namely if it prevents a subsequent growth of the biofilm, the remaining slides were left in the flow cells and were sampled 3 h after biocide application.

7.2.5 Analytical methods

7.2.5.1 Biofilm mass

The dry mass of the biofilm accumulated on the slides was assessed according to the procedure described in sub-chapter 3.6.5.

7.2.5.2 Respiratory activity

The respiratory activity of the several samples was evaluated by measuring the oxygen uptake rate (3.6.6).

7.2.6 Statistical analysis

The Student's *t* test was performed when the aim was to investigate whether the differences between the experimental values obtained under different conditions could be considered significant.

7.3 Results and discussion

7.3.1 Biofilm inactivation and removal after ortho-phthalaldehyde application

Figure 7.2 presents the percentage of biofilm inactivation measured immediately OPA application for both turbulent and laminar biofilms as a function of OPA concentration.

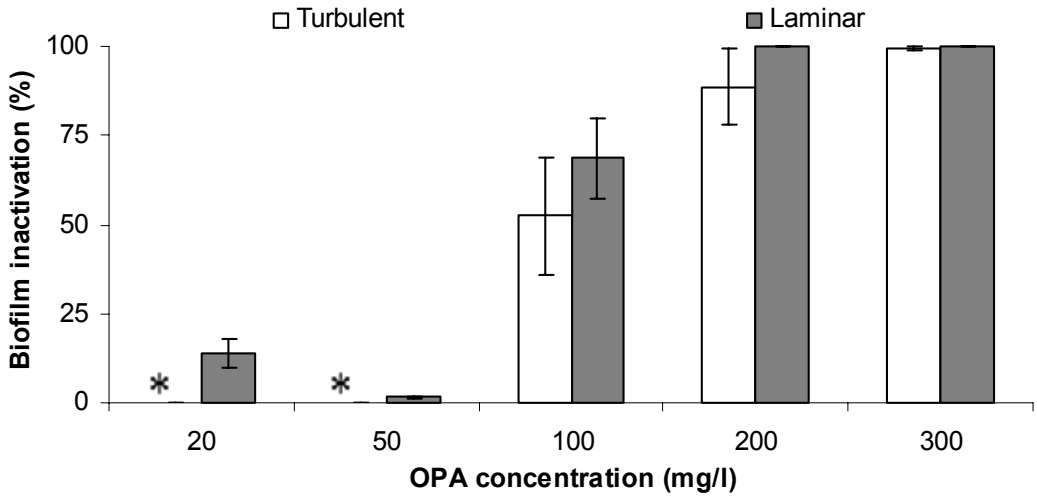


Figure 7.2 Biofilm inactivation as a function of OPA concentration.
(*) – The asterisks indicate that the biofilm was completely inactivated.

The increase in the OPA concentration applied to the biofilms enhanced its biocidal activity, both in turbulent and laminar biofilms, being this trend more consistent for OPA concentrations higher than 50 mg/l. The effect of the biocide seems to be dependent on the flow regime under which the biofilms were formed, since laminar biofilms were more easily inactivated ($P < 0.05$), specially in the cases when higher concentrations of OPA were used. Furthermore, only for laminar biofilms and higher biocide concentrations the total inactivation of the biofilm was achieved.

Figure 7.3 presents the respiratory activity of turbulent (Figure 7.3a) and laminar (Figure 7.3b) biofilms, immediately after OPA treatment and 3 h later.

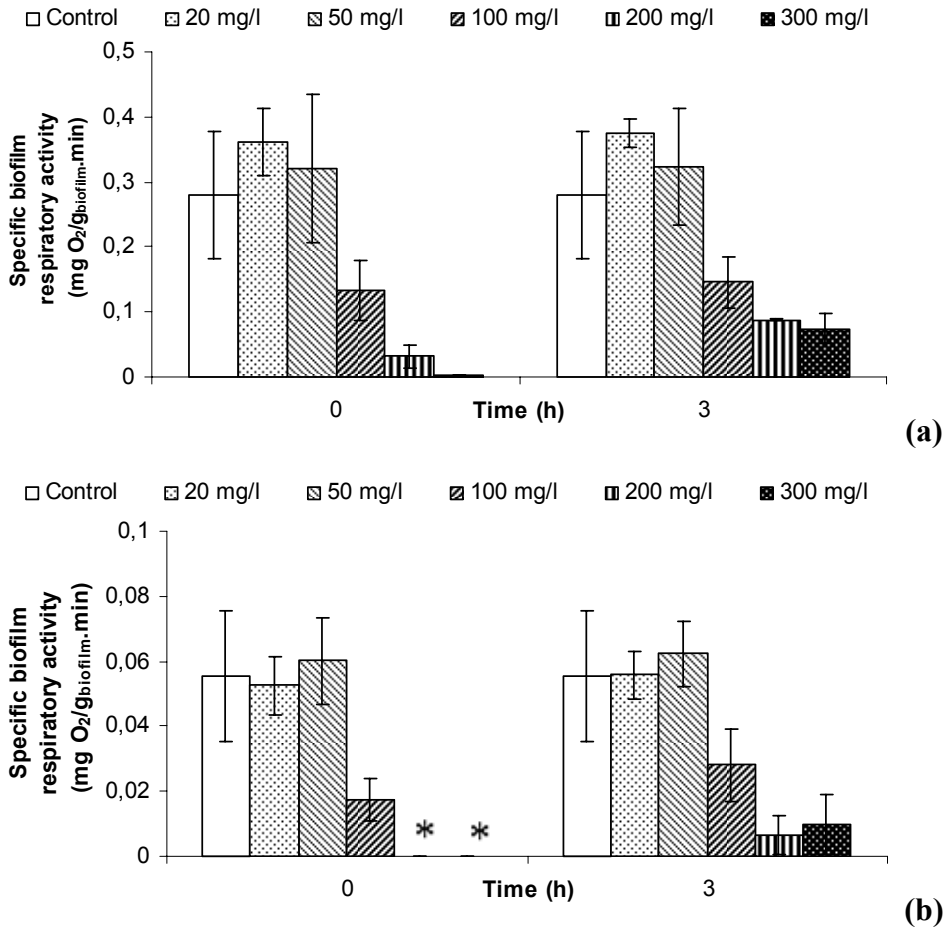


Figure 7.3 Biofilm activity immediately (0 h) after OPA treatment and 3 h later, for turbulent (a) and laminar (b) flow. Control means without OPA treatment.
(*) – The asterisks indicate that the specific biofilm respiratory activity was non-detectable.

Immediately after OPA application, an increase in OPA concentration decreased gradually the biofilm activity, both in turbulent and laminar flow (as above mentioned, the data is more consistent for OPA concentrations higher than 50 mg/l). Also, Figure 7.3a and 7.3b denote that 3 h after OPA application, for the biofilms that contact with higher OPA concentrations, and for both flow regimes, it seems that there is an increase in the biofilm activity compared to time zero, suggesting that bacterial biofilms recovered, in some extent, the respiratory activity.

Figure 7.4 presents the percentage of biofilm that remained adhered after chemical treatment measured immediately OPA application for both turbulent and laminar biofilms as a function of OPA concentration.

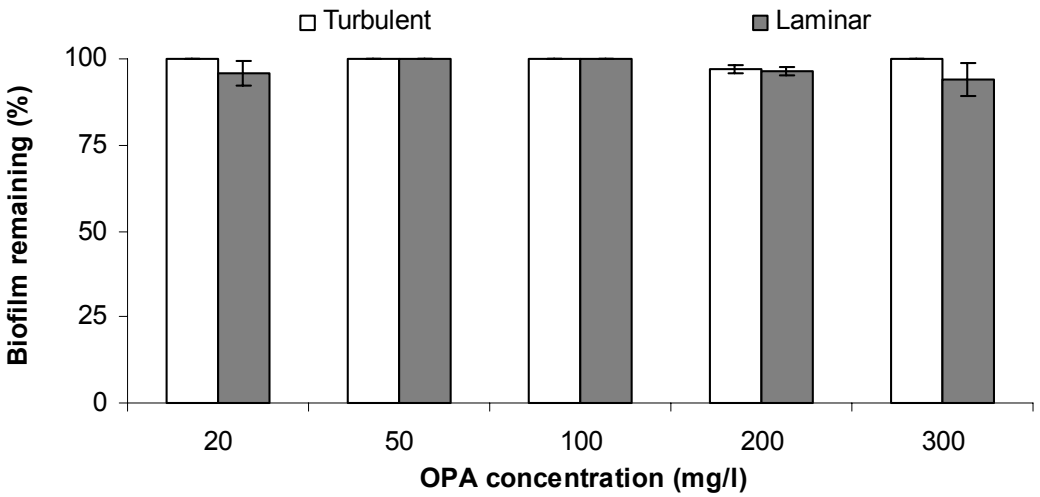
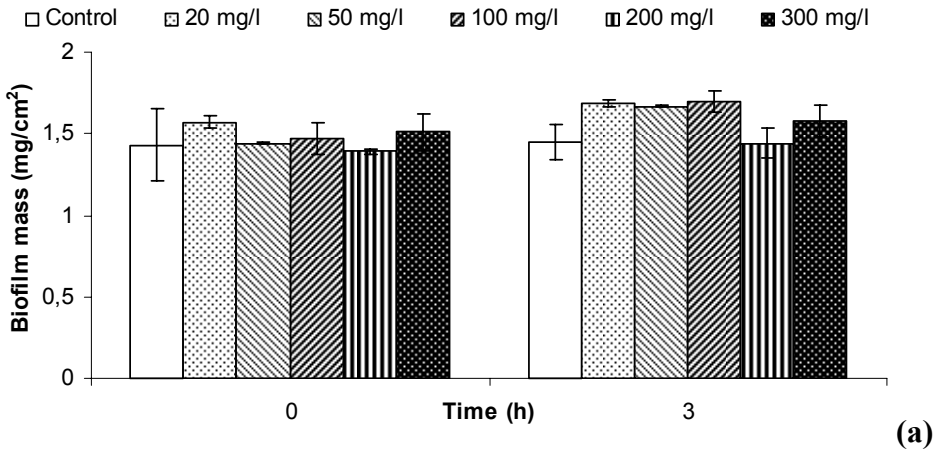


Figure 7.4 Biofilm remaining as a function of OPA concentration.

From this result (Figure 7.4), it can be concluded that OPA does not promote the detachment of the biofilms from the surface ($P > 0.1$).

In order to assess if the biocide had a more prolonged effect on post-biocide application, Figure 7.5 presents the biofilm mass of turbulent (Figure 7.5a) and laminar (Figure 7.5b) biofilms, after treatment with different OPA concentrations and 3 h later.



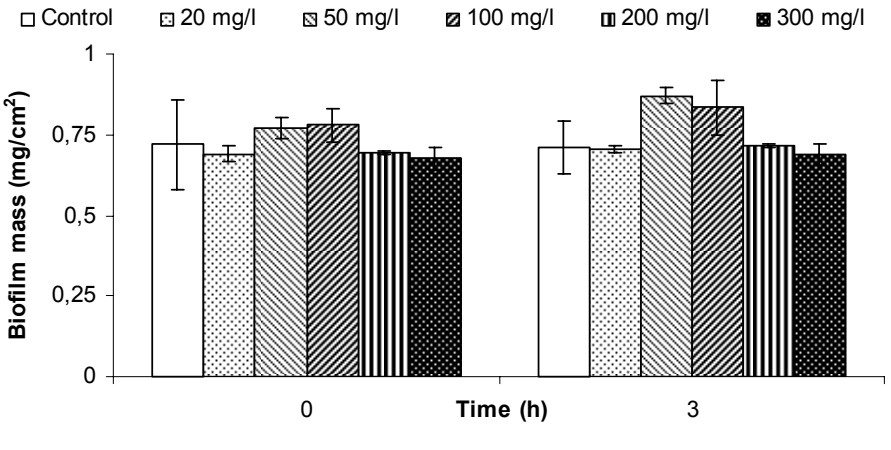


Figure 7.5 Biofilm mass after OPA treatment and 3 h later, for turbulent (a) and laminar (b) flow, after application of different concentrations of OPA. Control means without OPA treatment.

The biocide seems to have no effect on the variation of biofilm mass for the range of conditions studied, in view of the fact that the mass of the different turbulent and laminar biofilms did not experience any representative variation due to OPA treatment, for both sampling times ($P > 0.1$).

7.3.2 Biofilm inactivation and removal after glutaraldehyde application

The action of GTA, immediately after application, in the metabolic activity of turbulent and laminar biofilms can be seen in Figure 7.6.

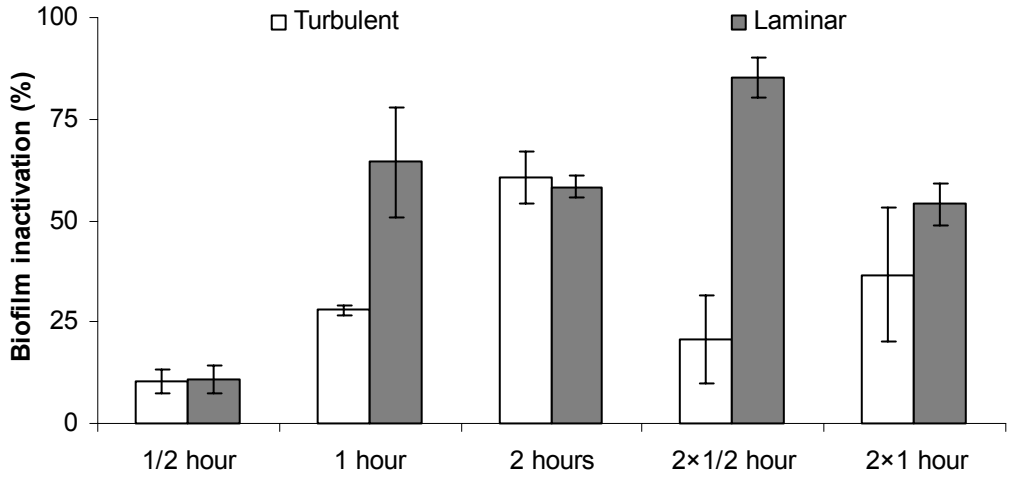


Figure 7.6 Biofilm inactivation as a function of exposure time to GTA.

GTA proved to be more effective in the inactivation of biofilms for longer exposure times (Figure 7.6). For the same total exposure time, the multiple application of biocide proved to be less efficient than the application in a single period of time. The flow regime under which the biofilm is formed seems to have a strong impact on the biocide action, since biofilms formed under laminar flow are more easily inactivated than the ones formed under turbulent flow ($P < 0.05$). However, total inactivation of biofilms was not achieved for both turbulent and laminar biofilms.

Figure 7.7 shows the respiratory activity, for turbulent (Figure 7.7a) and laminar (Figure 7.7b) biofilms, immediately after GTA treatment and 3 h later.

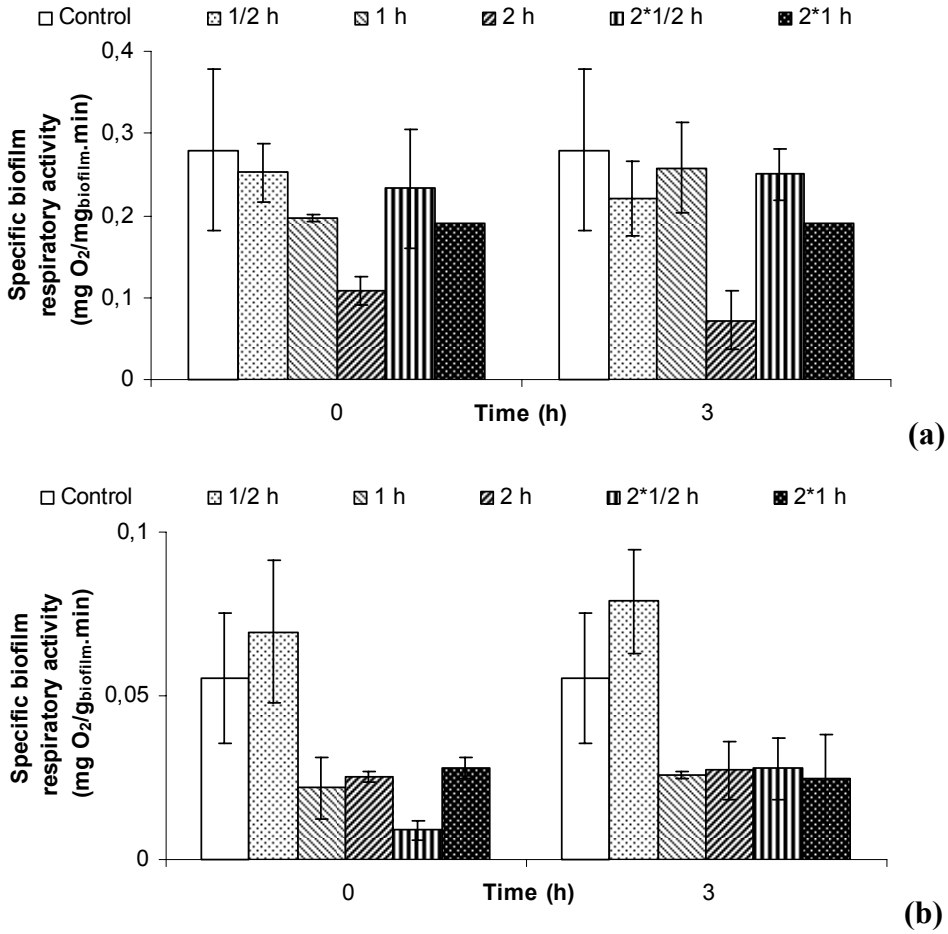


Figure 7.7 Biofilm activity after GTA treatment and 3 h later, for turbulent (a) and laminar (b) flow. Control means without GTA treatment.

Three hours after GTA application, biofilm activity seemed not to be significantly changed for every exposure time studied and for both biofilms ($P > 0.05$).

Figure 7.8 presents the percentage of biofilm remaining after chemical treatment, due to the application of 200 mg/l of GTA during different exposure periods, for turbulent and laminar flow.

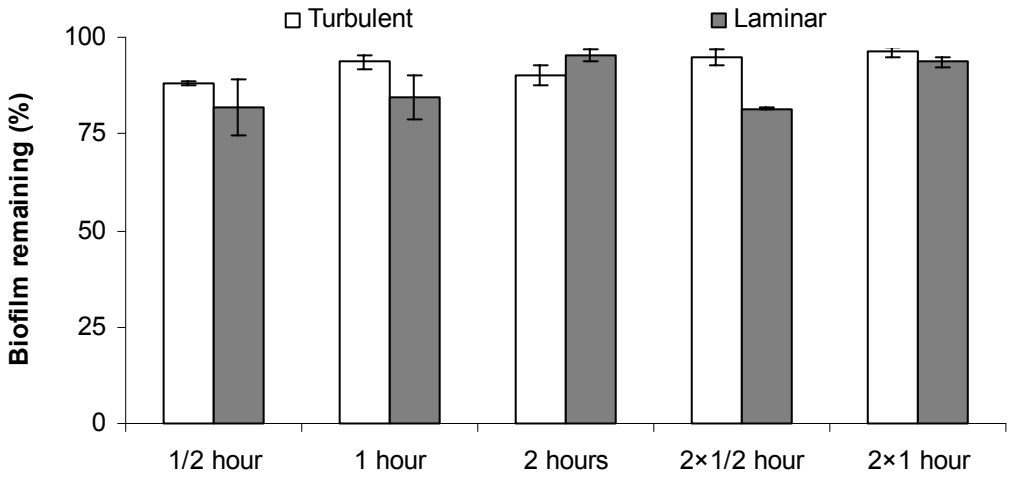
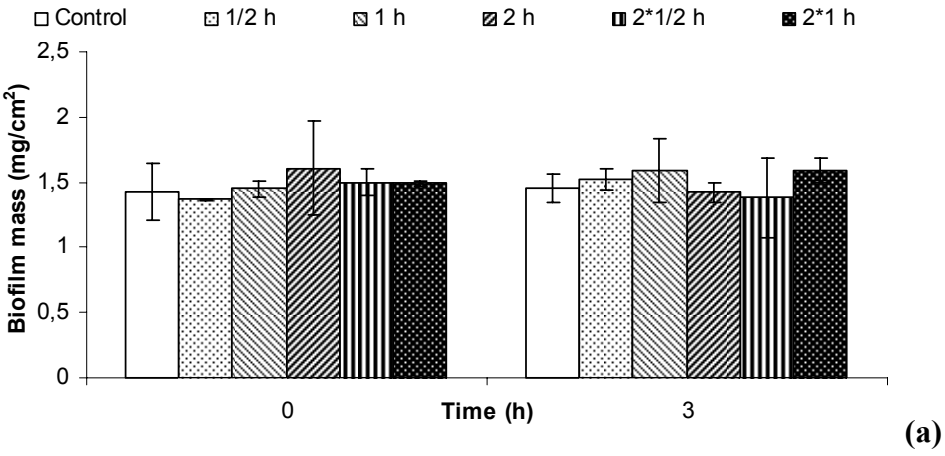


Figure 7.8 Biofilm remaining as a function of exposure time to GTA.

Figure 7.8 shows that biofilms were not removed after the GTA treatment, in all situations studied ($P > 0.1$).

Figure 7.9 shows the biofilm mass, of turbulent (Figure 7.9a) and laminar (Figure 7.9b) biofilms, immediately after GTA treatment and 3 h later.



(a)

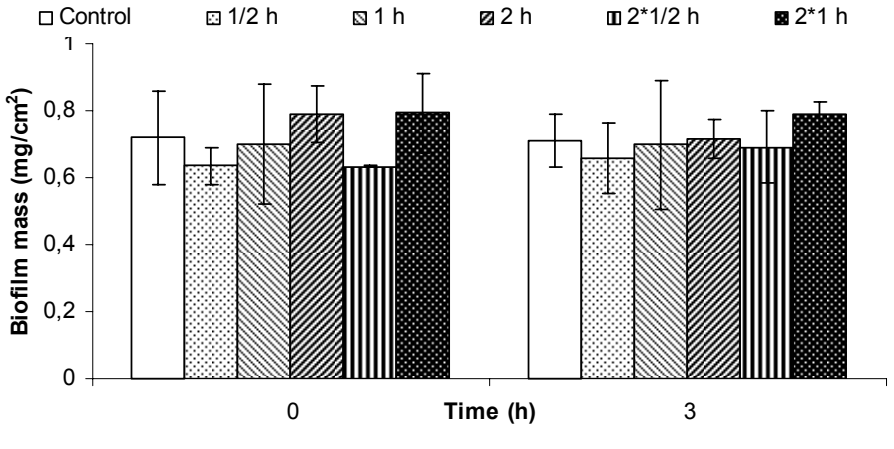


Figure 7.9 Biofilm mass after GTA treatment and 3 h later for turbulent (**a**) and laminar (**b**) flow. Control means without GTA treatment.

Three hours after GTA application, the mass of the biofilm did not changed for every exposure time studied ($P > 0.1$).

7.3.3 Comparison between ortho-phthalaldehyde and glutaraldehyde against biofilms – influence of the flow regime and cellular state

Since the hydrodynamic conditions under which the biofilms are formed play a significant role in the composition and structure of the biofilms (Chapter 5), it is not surprising to find differences on the efficacy of biocides when applied to turbulent and laminar biofilms. In all the situations studied, the laminar biofilms were more susceptible to the biocide action than the turbulent biofilms. Several reasons may account for this behaviour as already reported by several authors: apart from diffusion limitations that may occur, the penetration of antimicrobial agents into microbial biofilms is also controlled by the reaction of the antimicrobial agent with biofilm components (Stewart *et al.*, 2001), since the biocide can react with the community components, such as organic matter, inorganic particles and cell debris (McFeters *et al.*, 1995); the exopolymeric matrix is charged, being responsible for binding antimicrobial agents before they reach the target cell (Costerton, 1985); phenotypic characteristics cells within biofilms are different from that in planktonic state as previously demonstrated in Chapter 5. Therefore, when a biocide is used to control biofilms, the microbial response to the chemical agent will depend not only on the type of microorganisms and the type of chemical agent, but also on the complex interactions between the biocide and the cellular arrangement. The higher content of proteins and

biofilm mass present in biofilms formed under turbulent flow may have reacted with the biocides, lowering their concentrations and thus reducing their antimicrobial effect. In previous work, Pereira and Vieira (2001) already suggested that GTA, when applied against biofilms, reacted with proteins of the polymeric matrix besides the reaction with the bacterial cells. As a consequence, the concentration of the biocide available for reaction with the bacteria within the biofilm is reduced. The results obtained in suspended tests in the presence of BSA (Chapter 6) reinforce this last statement.

Comparing the action of OPA and GTA, when applied at the same concentration and exposure time (Table 7.1) to cells within biofilms and planktonic cells (results from Chapter 6), both biocides proved to be more efficient in *P. fluorescens* suspended cultures than against the bacterial biofilms, emphasizing that bacteria entrapped in a biofilm are more resistant to antimicrobial agents than freely suspended cells (Table 7.1).

Table 7.1 Bacterial inactivation in the presence and absence of BSA and inactivation of biofilms formed under turbulent and laminar flow due to the application of several concentrations of OPA and 200 mg/l of GTA during ½ h. Mean ± SD

	Planktonic cells		**Biofilm**	
	Without BSA	With BSA	Turbulent	Laminar
OPA				
200 mg/l	100 ± 0	90.2 ± 5.5	88.6 ± 11	100 ± 0
GTA				
200 mg/l	48.8 ± 2.9	5.82 ± 0.30	10.3 ± 2.8	10.8 ± 3.5

Comparing the action of OPA and GTA in the inactivation of *P. fluorescens* under different cellular states, OPA revealed to be more efficient than GTA.

Concerning the analysis of the results obtained for the biofilms 3 h (post-surfactant application) after OPA treatment, shows that the respiratory activity is always higher than the one achieved immediately after biocide treatment, regardless of the flow regime, while for GTA the activity of the biofilm seems to continue invariable. This fact can be due to the higher level of cross-linking promoted by GTA (Eager and Leder, 1986; Walsh *et al*., 1999a; 1999b; Simons *et al.*, 2001) that can have a prolonged effect on post-biocide application and therefore in the biofilm development.

Table 7.2 shows the differences obtained in biofilm removal after a similar process of treatment, with OPA and GTA.

Table 7.2 Removal of biofilms formed under turbulent and laminar flow due to the application of 200 mg/l of OPA and GTA during ½ h. Mean ± SD

	Biofilm removal (%)	
	Turbulent	Laminar
OPA	2.90 ± 1.2	3.6 ± 1.1
GTA	11.9 ± 0.35	18.1 ± 7.1

The effect of both biocides on the biofilm mass was similar, *i.e.*, the application of OPA and GTA did not promote a significant biofilm removal. Hence, the biofilms can be inactive but stay attached to the surface, which is not convenient in industrial systems where biofilm accumulation is a problem and, as seen above, the remaining biofilm can restore its functions along time.

7.4 Conclusions

The results presented in this chapter showed that OPA and GTA were more effective in the inactivation of laminar biofilms than turbulent biofilms, suggesting that the flow regime under which the biofilm are formed play an important role in the biocide action, specially when extremes conditions are tested (higher biocide concentrations or exposure times). Contrary to OPA, the application of GTA did not promote total inactivation of biofilms.

When analysing the mode of application of biocides, the increase in the biocide concentration seems to be more efficient than the increase in the time of contact of the biocide with the biofilm.

Both biocides exhibited a poor ability for the removal of laminar or turbulent biofilms from the surfaces.

It can be concluded that biofilms are very stable structures that can be inactivated but stay attached to the surfaces. Both biocides proved to be more efficient in *P. fluorescens* suspended cultures than against the bacterial biofilms, emphasizing that bacteria entrapped in a biofilm are more resistant to antimicrobial agents than suspended cells, showing the inadequacy of planktonic testing methods for evaluating antimicrobial agents to be used against biofilms, and the need to use biofilm testing methods.

The chemical structure of the biocide seemed to have influence in the potential of the biofilms to recover their metabolic activity since, 3 h after the treatment with OPA, the biofilms appear to recover their metabolic activity, while the same effect did not occurred after GTA treatment.

Chapter 8

Action of surfactants on the inactivation and removal of biofilms formed by *Pseudomonas fluorescens* under different flow regimes

Abstract

The action of the CTAB and SDS are investigated in the control of biofilms formed on stainless steel slides, under turbulent and laminar flow, by *P. fluorescens*. The contact of the surfactants with the biofilms is performed during ½ h. The action of different concentrations of the surfactants on biofilms is assessed by means of cellular respiratory activity and variation of biofilm mass, immediately, 3, 7 and 12 h after the application of the surfactants. The latter times are tested in order to ascertain a possible prolonged effect on post-surfactant application. Before and after the chemical treatment, scanning electron microscopy analyses are carried out in order to assess the influence of the surfactants on the biofilm structure.

8.1 Introduction

Surfactants are commonly used in mixtures of cleaning products because of their ability to wet surfaces, penetrate soil and solubilize fatty materials (Glover *et al.,* 1999; Christofi and Ivshina, 2002). Surfactants are classified on the basis of the charge or absence of ionization of the hydrophilic group (McDonnell and Russell, 1999). Cationic surfactants or QAC´s are employed both as disinfectants for manual processing lines and surfaces in the food industry, and in human medicine area (Mereghetti *et al.,* 2000), because of their excellent hard-surface cleaning, deodorization and antimicrobial properties (McDonnell and Russell, 1999). QAC´s mode of action is attributed to their positive charge, which forms an electrostatic bond with negatively charged sites on microorganisms cell walls (McDonnell and Russell, 1999). Those electrostatic bonds create stresses in the wall, leading to cell lysis and death. The QAC´s also cause cell death by protein denaturation, distortion of cell-wall permeability and reduction of the normal intake of life-sustaining nutrients to the cell (Cloete *et al.* 1998). CTAB is a QAC that appears to rupture the cell membrane. The primary site of action of CTAB has been suggested to be the lipid components of the membrane being cell lyses a second effect (Gilbert *et al.* 2002).

Anionic surfactants possess strong detergent but weak antimicrobial properties, except at high concentrations, when they induce lyses of Gram-negative bacteria (Glover *et al.,* 1999). The outer and cytoplasmic membranes and the membrane-bound enzyme environment and function are the main targets of anionic surfactants (Cloete *et al.,* 1998).

The purpose of the work presented on this chapter is to assess the efficacy of CTAB and SDS to control biofilms of *P. fluorescens* formed under turbulent and laminar flow, and to evaluate the capability of the biofilms to recover after chemical treatment.

8.2 Material and methods

8.2.1 Microorganism and culture conditions

The microorganism used was *P. fluorescens* ATCC 13525^{T}. The culture conditions were followed according to the procedure described in sub-chapter 3.1.2.

8.2.2 Biofilm system

The continuous flow cell system (sub-chapter 3.3) was used to perform biofilm formation under turbulent and laminar flow.

8.2.3 Surfactants

CTAB was used at 0.125, 0.250, 0.500 and 0.900 mM.

SDS was used at 0.500, 1.00, 3.00 and 7.00 mM.

Surfactant solutions were diluted to the required concentration with sterile water.

8.2.4 Biofilm tests

The biofilms formed on the metal slides of each parallel flow cell reactor were exposed to different concentrations of surfactant for ½ h. Each surfactant concentration was tested in an independent experiment and each experiment was performed on three separate occasions. During the treatment period, the surfactant solution replaced the diluted bacterial suspension flowing in the flow cell reactors, as already pointed out in sub-chapter 7.2.4. After the exposure time to the surfactant, the flow of the surfactant solution through the flow cells was stopped and the bacterial suspension was re-introduced in the system, as described in sub-chapter 7.2.4. In each experiment, and prior to the beginning of the surfactant treatment, two metal slides of each flow cell operating in parallel were sampled and used as a control. Immediately after the surfactant treatment, two metal slides of each flow cell were also sampled (time zero). The biofilms that covered the stainless steel slides were completely scraped, the residual surfactant was neutralized (sub-chapter 3.5.1) and the biofilm was used for further analysis according to the experiment describe in sub-chapter 7.2.4. In order to assess whether time plays a significant role on the action of surfactant, namely if it prevents a subsequent growth of the biofilm, the remaining slides were left in the flow cells, without neutralization of the surfactant, and were sampled 3, 7 and 12 h after surfactant application. For every condition tested and for all times of exposure, two stainless steel slides were sampled.

8.2.5 Analytical methods

8.2.5.1 Biofilm mass

The dry mass of the biofilm accumulated on the slides was assessed according to the procedure described in sub-chapter 3.6.5.

8.2.5.2 Respiratory activity

The respiratory activity of the several samples was evaluated by measuring the oxygen uptake rate (sub-chapter 3.6.6).

8.2.5.3 Scanning electron microscopy observations

During the experiments, several stainless steel slides covered with biofilms were observed by SEM (sub-chapter 3.6.10).

8.2.5.4 Epifluorescence microscopy

A volume of 50 μL of the biofilm suspension was stained with L-7012 Live/Dead® *Bac*Light™ Bacterial Viability kit developed by Molecular Probes Inc., using epifluorescence microscopy according to the procedure described in sub-chapter 4.2.6. The biofilm suspension was visualized before, after chemical treatment and 12 hours later.

8.2.6 Statistical analysis

Statistical comparisons of biofilm inactivation, biofilm removal and recovery were analysed by Student's *t* test.

8.3 Results and discussion

8.3.1 Biofilm inactivation and removal after cetyltrimethyl ammonium bromide application

The effects of the application of CTAB during ½ h against biofilms formed on stainless steel slides under turbulent and laminar flow was assessed either by determining the respiratory activity due to glucose oxidation and the variation of the mass of biofilm. Those results are presented in terms of percentage of biofilm inactivation and removal (Figures 8.1 and 8.2), immediately after CTAB application.

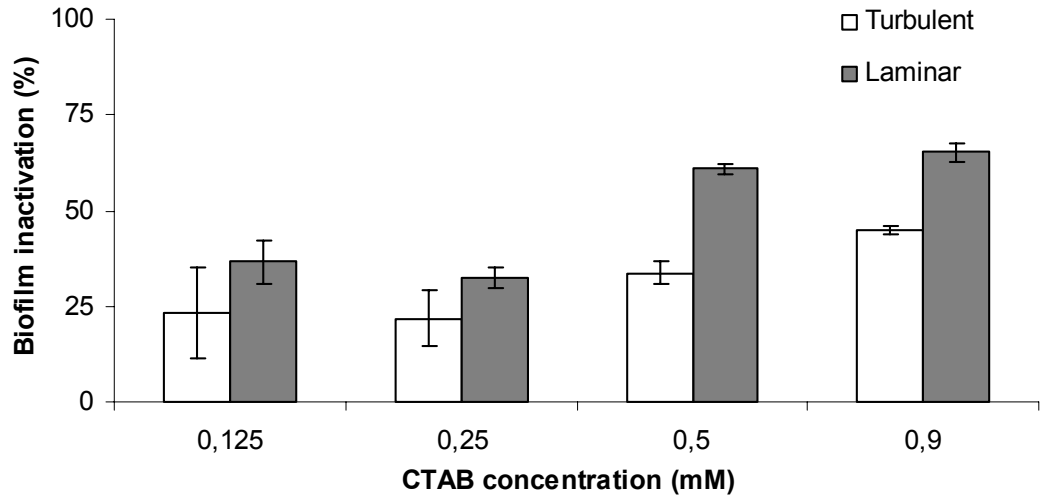


Figure 8.1 Biofilm inactivation as a function of CTAB concentration.

The application of several concentrations of CTAB to biofilms formed in the flow cell reactors resulted in an inactivation of the bacteria within the biofilm, which increased with the increase of the surfactant concentration (Figure 8.1). Concerning the studies carried out with biofilms formed under different flow regimes, the inactivation effect of CTAB was more pronounced in laminar biofilms than turbulent biofilms ($P < 0.05$). Nevertheless, total biofilm inactivation was not achieved for every condition studied. From these results, it can be said that the development of successful strategies to control biofilm formation must be studied under conditions that mimic real situations, since biofilm properties change in response to environmental conditions (Pereira *et al.* 2002b; Vieira *et al.* 1993). Biofilms formed under laminar flow are easily inactivated than those formed under turbulent flow.

The understanding of the effect of operational parameters that affect biofilm formation and subsequent disinfection plays a basic role on the establishment of a biofilm

control program. The low efficacy of CTAB to control biofilms may be related with its chemical reaction with proteins of the exopolymeric matrix. This argument is reinforced by the tests carried out with planktonic cells (Chapter 6), which showed that the inactivation effect of CTAB was significantly reduced in the presence of BSA. The higher inactivation effect on laminar biofilms is probably related with the characteristics of laminar biofilms when compared with the turbulent ones (Chapter 5). However, in both hydrodynamic situations, problems associated with mass transfer limitations within the biofilms can, always, decrease the action of CTAB.

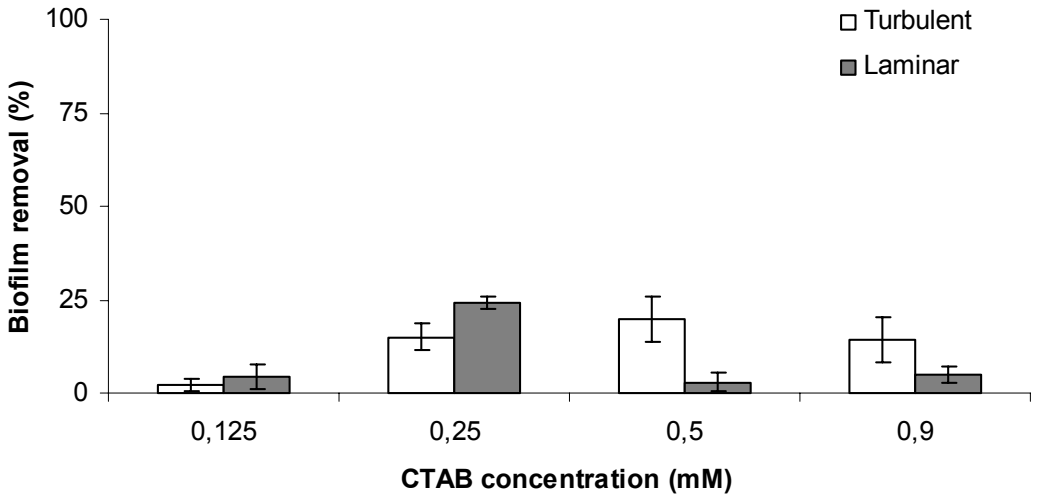
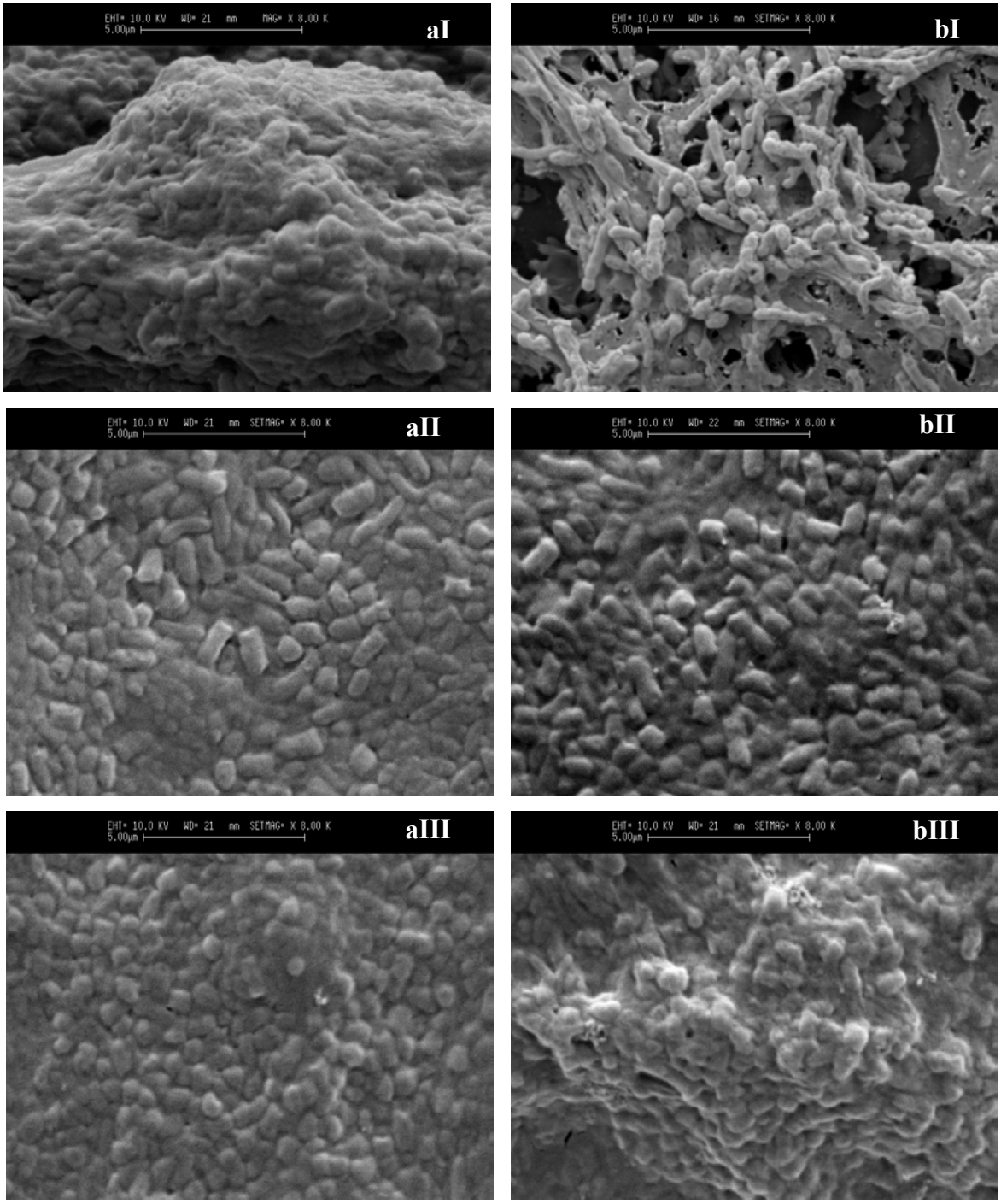


Figure 8.2 Biofilm removal as a function of CTAB concentration.

Concerning biofilm removal (Figure 8.2), CTAB had not a significant effect since the biofilm removal was always less or similar than 25 %, independently of the CTAB concentration. For laminar biofilms, the higher detachment was induced by a concentration of 0.250 mM, while for turbulent biofilms it was achieved only for a concentration of 0.5 mM. Comparing statistically the percentage of biofilm removal for turbulent and laminar biofilms the results are similar ($P > 0.1$).

The results show that the ability of CTAB to inactivate the biofilm is higher than to remove biofilms from surfaces, leaving biofilm on the surface not fully inactivated. Azeredo *et al.* (2003b) already showed that CTAB (0.5 mM) had the ability to cement bacteria to glass in spite of removing them. Despite the low effect on the biofilm removal, SEM observations reveal that the biofilm structure is changed after CTAB application (Figure 8.3).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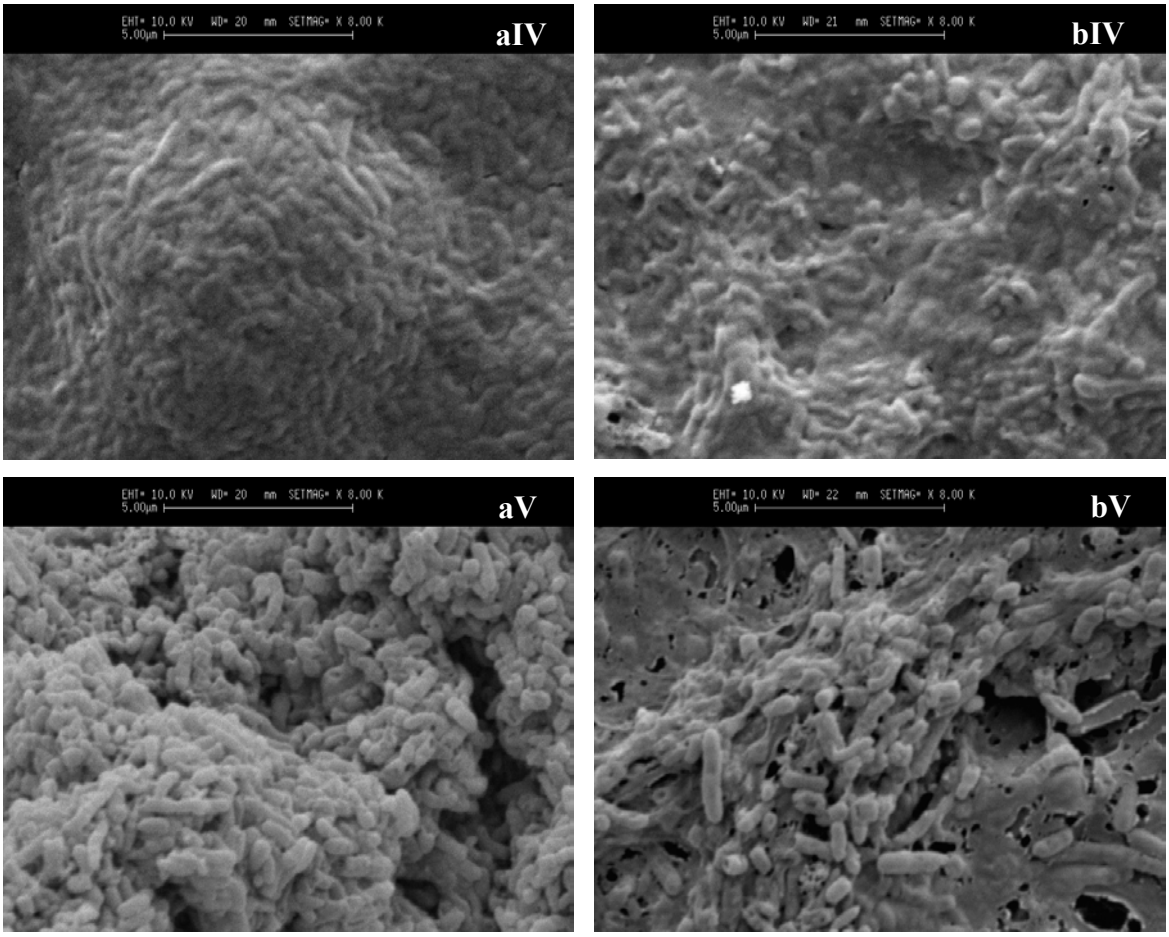


Figure 8.3 Scanning electron microscopy microphotographs of a 7 d old *P. fluorescens* biofilms formed on stainless steel slides under turbulent (**a**) and laminar flow (**b**) without surfactant application (**I**) and after treatment with 0.125 (**II**), 0.500 (**III**), 1.00 (**IV**) and 0.900 mM (**V**) of CTAB during ½ h. X 8000 magnification, bar = 5 μm.

SEM observations show that biofilms formed under different flow regimes present significant morphological differences and that CTAB caused damage in the structure of the bacterial biofilms, being this phenomenon more pronounced with the application of 0.125, 0.250 and 0.500 mM.

8.3.2 Biofilm inactivation and removal after sodium dodecyl sulfate application

Results of biofilm inactivation and biofilm removal after treatment with SDS at several concentrations are plotted in Figures 8.4 and 8.5.

SDS promoted biofilm inactivation, being this effect dependent of the concentration. However, in the range of concentrations tested, total inactivation was not achieved.

Comparing the results obtained for the turbulent and laminar biofilms, the statistical analysis showed that both biofilms had similar susceptibility to SDS action ($P > 0.1$).

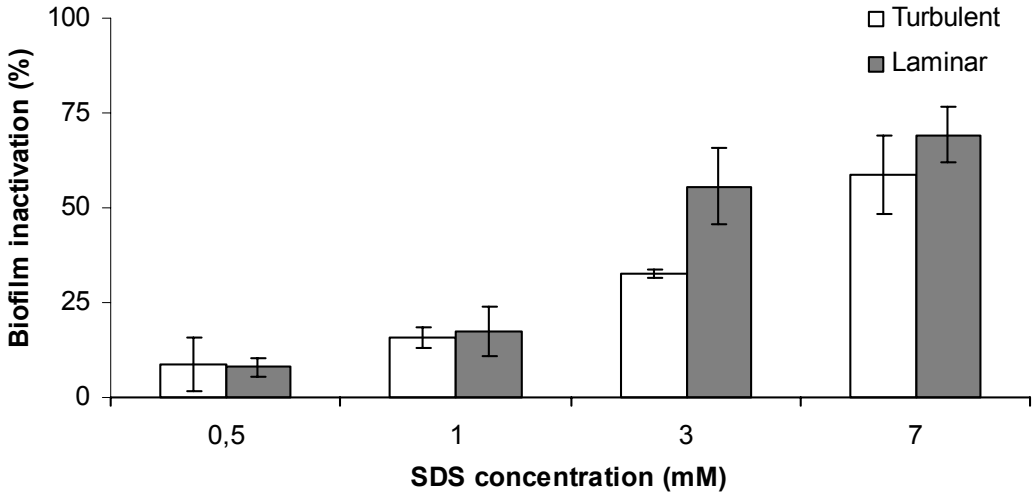


Figure 8.4 Biofilm inactivation as a function of SDS concentration.

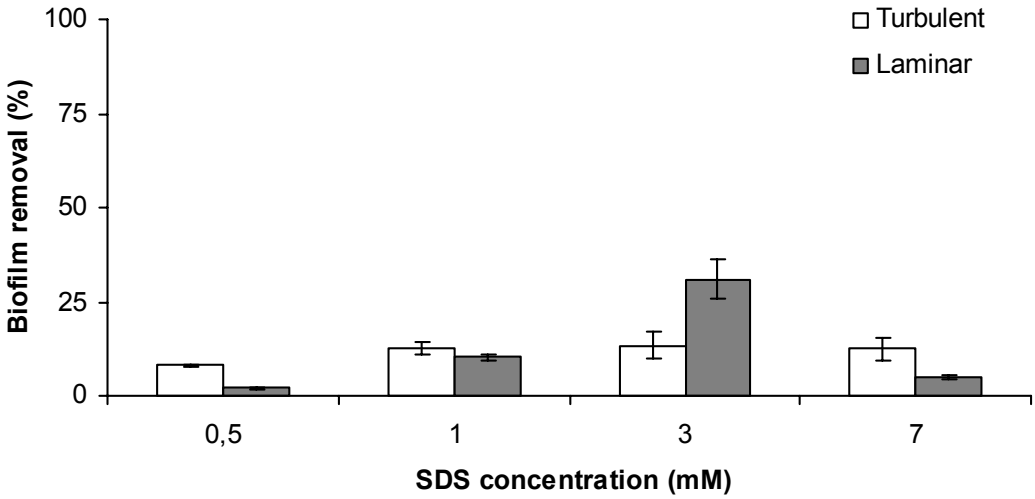


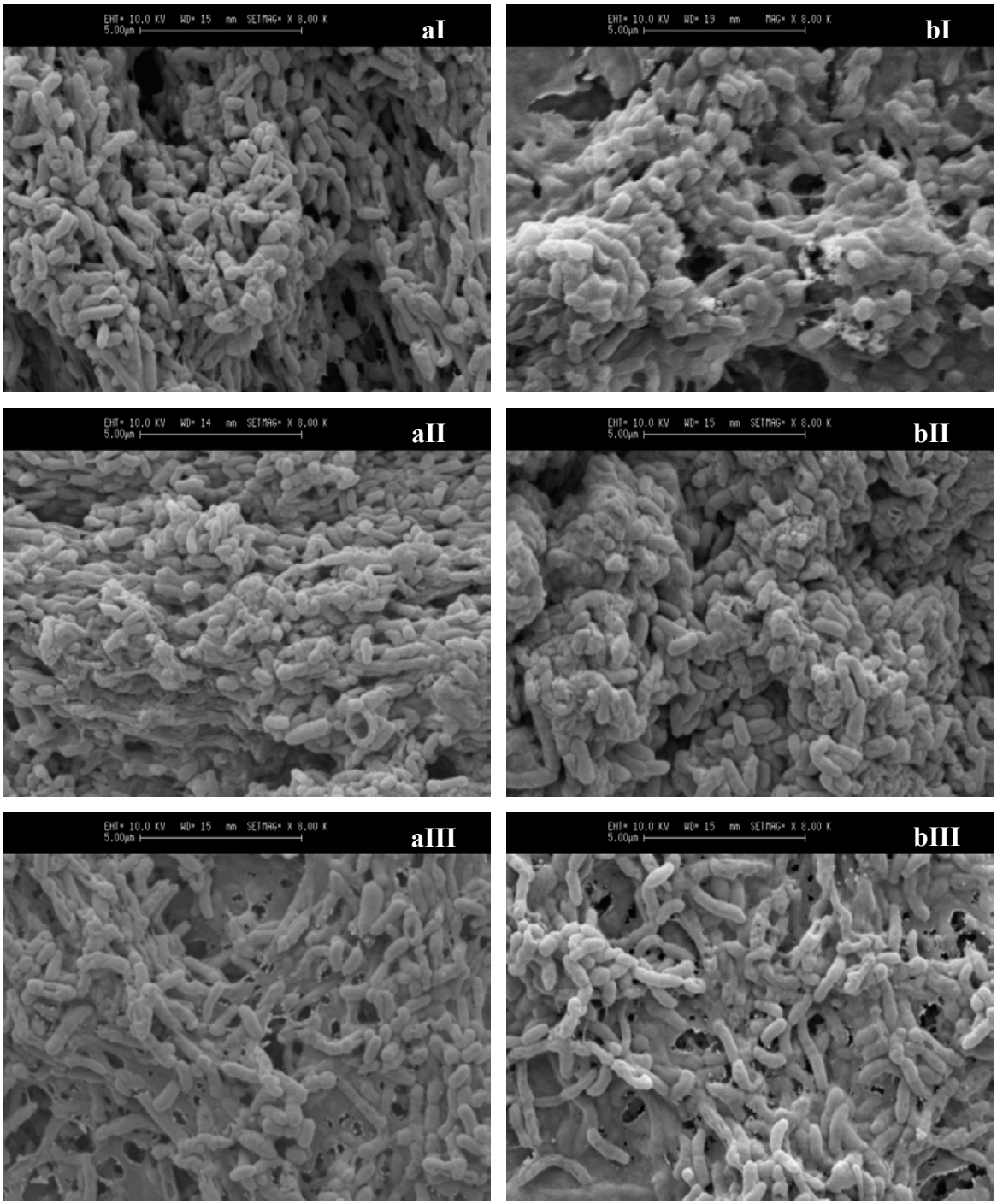
Figure 8.5 Biofilm removal as a function of SDS concentration.

Concerning biofilm removal, SDS had a poor effect on the biofilm removal, for both biofilms, since in almost all the experiments (except for 3 mM and for biofilm formed under laminar flow) the biofilm removal was less than 20 %. The removal was not dependent on the surfactant concentration, since the increase in the SDS concentration did not increased the biofilm removal. Furthermore, the statistical analysis revealed no equivalence on the removal of biofilms formed under different flow regimes ($P < 0.05$).

Previous studies made by Azeredo *et al.* (2003b) demonstrated that SDS was efficient in the removal of layers of cells attached to glass. However, in this work, it was

found a lack of biofilm susceptibility to SDS, in terms of biofilm removal. Concerning the difference found within biofilms, it reflects the impact of the flow regime under which the biofilm are formed in the posterior biofilm removal. Purevdorj *et al.* (2002) found for *P. aeruginosa*, that high shear flow leads to a formation of strongly adhered biofilms.

The evidence of bacterial biofilm in the metal slides before the treatment and the possible damage resulting from SDS treatment was inspected by SEM, as displayed in Figure 8.6.



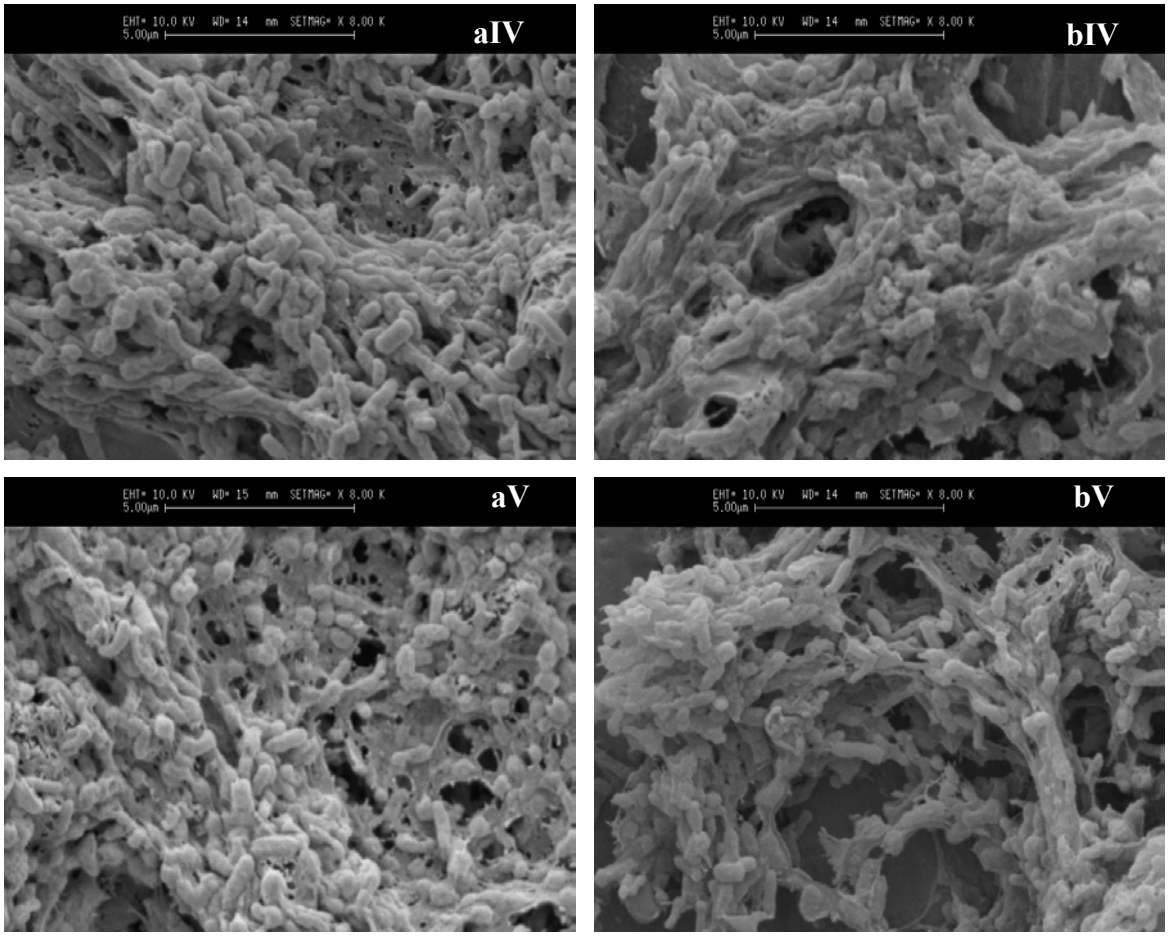


Figure 8.6 Scanning electron microscopy microphotographs of a 7 d old *P. fluorescens* biofilms formed on stainless steel slides under turbulent (**a**) and laminar flow (**b**) without surfactant application (**I**) and after treatment with 0.5 (**II**), 1 (**III**), 3 (**IV**) and 7 mM (**V**) of SDS during ½ h. X 8000 magnification, bar = 5 μm.

SEM observations show that biofilms formed under different flow regimes present significant morphological differences, as already stated in Chapter 5, and that SDS seemed to cause damage in the structure and integrity of the bacterial biofilms. The probable phenomenon behind this fact is related with reaction of SDS with the biofilm cells and the removal of layers of biofilm. For the treatment with 3 mM and for laminar biofilms it is clear the reduced amount of biofilm, which is in accordance with the result found for the biofilm removal, where this value is higher for this condition.

8.3.3 Comparison between cetyltrimethyl ammonium bromide and sodium dodecyl sulfate against biofilms – influence of the flow regime and cellular state

Table 8.1 shows results of suspended (already presented in Chapter 6) and biofilm bacteria inactivation in order to emphasize the role of the environmental conditions under study and the cellular state on the antimicrobial action of CTAB and SDS.

Table 8.1 – Bacterial inactivation in the presence and absence of BSA and inactivation of biofilms formed under turbulent and laminar flow due to the application of several concentrations of CTAB and SDS during ½ h. Mean ± SD.

	Planktonic cells		**Biofilm**	
	Without BSA	**With BSA**	**Turbulent**	**Laminar**
CTAB				
0.125 mM	59.0 ± 1.5	16.2 ± 2.3	23.3 ± 12	36.5 ± 5.7
0.250 mM	72.2 ± 3.1	24.3 ± 0.33	21.8 ± 7.3	32.3 ± 2.7
0.500 mm	100 ± 0	28.0 ± 1.8	33.6 ± 2.9	60.9 ± 1.4
0.900 mM	100 ± 0	86.8 ± 6.3	45.0 ± 1.0	65.2 ± 2.3
SDS				
0.500 mM	0 ± 0	0 ± 0	8.80 ± 6.9	8.00 ± 2.4
1.00 mM	5.47 ± 3.9	0 ± 0	15.7 ± 2.9	17.3 ± 6.4
3.00 mm	72.1 ± 6.1	0 ± 0	32.6 ± 0.95	55.7 ± 10
7.00 mM	87.7 ± 3.8	8.15 ± 3.2	58.8 ± 10	69.1 ± 7.4

Comparing the action CTAB and SDS in the control of the *P. fluorescens* biofilms, both surfactants did not promote total biofilm inactivation, being CTAB more effective than SDS for the lower (0.125 and 0.250 mM) concentrations tested. Comparing inactivation of cells in suspension and cells within biofilms, it can be seen that cells within biofilms are often less susceptible to both surfactants than cells grown in suspension, which shows a significant difference between biofilms when compared with their freely counterparts. Nevertheless, the antimicrobial effect of both surfactants against planktonic cells in the presence of BSA was significantly reduced, for values smaller than the biofilm situations, for SDS, showing the high reactivity of surfactants with proteins. The experiments with planktonic cells and with BSA (Chapter 6) suggest that the proteins existent in the biofilm matrix are not in an immediate and reactive form in such way that halts the surfactant action. Ionic charge interactions between the biofilm matrix and the surfactant, in the case

of SDS, may occur in addition to the existence of diffusion limitations (Costerton *et al.*, 1987). However, as previously reported (Chapter 5) the cellular arrangement and the environmental conditions under which biofilms are formed lead to particular phenotypic characteristics. This cellular feature, arguably, had impact in a context of biofilm/bacteria disinfection.

Table 8.2 shows the differences obtained in biofilm removal after a similar process of treatment, with CTAB and SDS.

Table 8.2 Removal of biofilms formed under turbulent and laminar flow due to the application of 0.500 mM of CTAB and SDS during ½ h. Mean ± SD.

	Biofilm removal (%)	
	Turbulent	**Laminar**
CTAB	19.9 ± 6.0	2.92 ± 2.5
SDS	8.00 ± 0.38	2.13 ± 0.24

The biofilm removal with SDS application seems to be negligible for both turbulent and laminar biofilms as already pointed out in sub-chapter 8.3.2. CTAB, for this concentration, promotes a small reduction in the mass of biofilm formed under laminar flow. Hence, as verified for GTA and OPA (Chapter 7), the biofilms can be inactive but stay attached to the surface, which is not convenient in industrial systems where biofilm accumulation is a problem and the remaining biofilm can restore its functions.

8.3.4 Biofilm recovery after treatment with cetyltrimethyl ammonium bromide and sodium dodecyl sulfate

Figure 8.7 presents the post-surfactant effect, in terms of respiratory activity, of turbulent (a) and laminar (b) biofilms, after CTAB treatment. That effect was evaluated after 3, 7 and 12 h later and compared with the results obtained immediately after the chemical treatment (0 h).

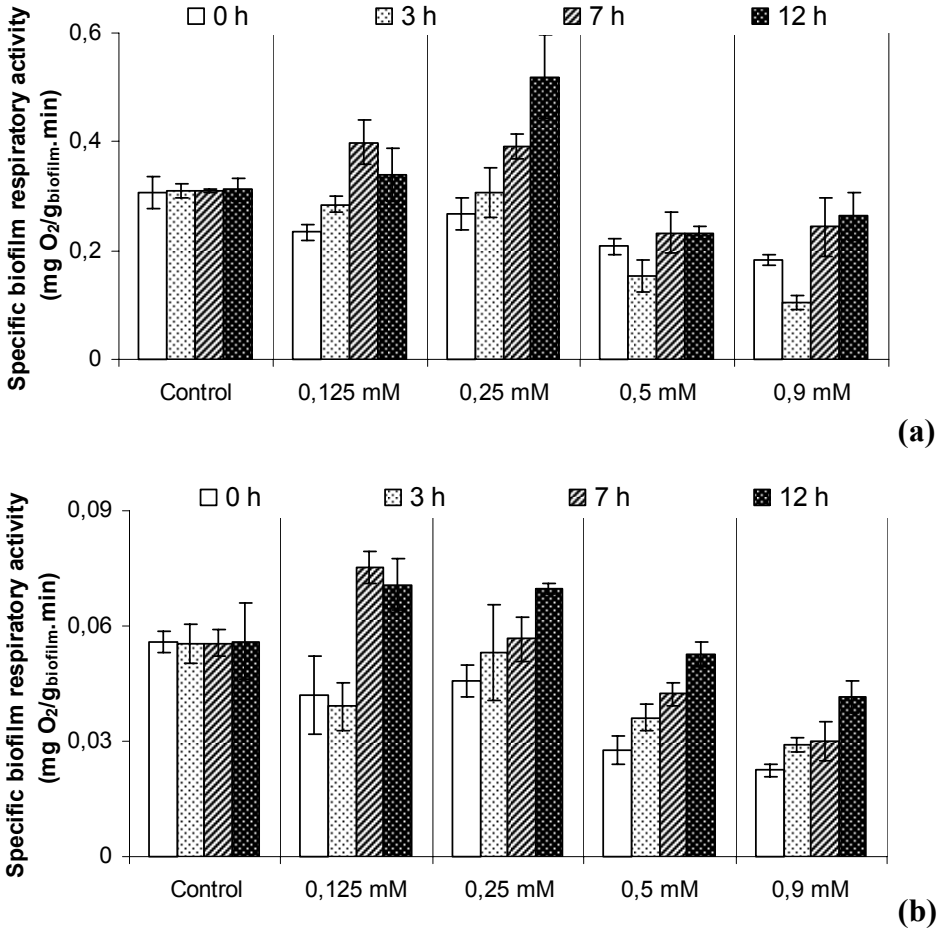


Figure 8.7 Biofilm respiratory activity after chemical treatment (0 h) with CTAB and 3, 7 and 12 h later for biofilms formed under turbulent (**a**) and laminar (**b**) flow. Control means without surfactant treatment.

The biofilms which were not immediately sampled after surfactant application were not subjected to the neutralization step being expected a sustained antimicrobial effect that promoted the failure of the cohesive forces of the biofilm, encouraging the consequent removal, since the surfactant retained within the biofilm matrix had more chance to act on the bacteria. Forsyth and Hayes (1998) stated that surfaces treated with cationic surfactants could retain a bacteriostatic film, due to the adsorption of the chemical on the surface, and prevent the subsequent growth of residual bacteria.

From the results obtained after treatment with CTAB, the respiratory activity increased with the time between CTAB application and biofilm sampling, reaching values higher than the ones observed in the control experiment, *i.e.,* without surfactant application. Both turbulent and laminar biofilms have similar recovery profiles when comparing statistically ($P > 0.05$). The control experiments show that the biofilm activity was almost

independently of the time ($P > 0.05$) since the 7 d old biofilms exhibit the same respiratory activity during the time of experiment (12 h).

Figure 8.8 presents the post-surfactant effect, in terms of respiratory activity, of turbulent (a) and laminar (b) biofilms, after SDS treatment.

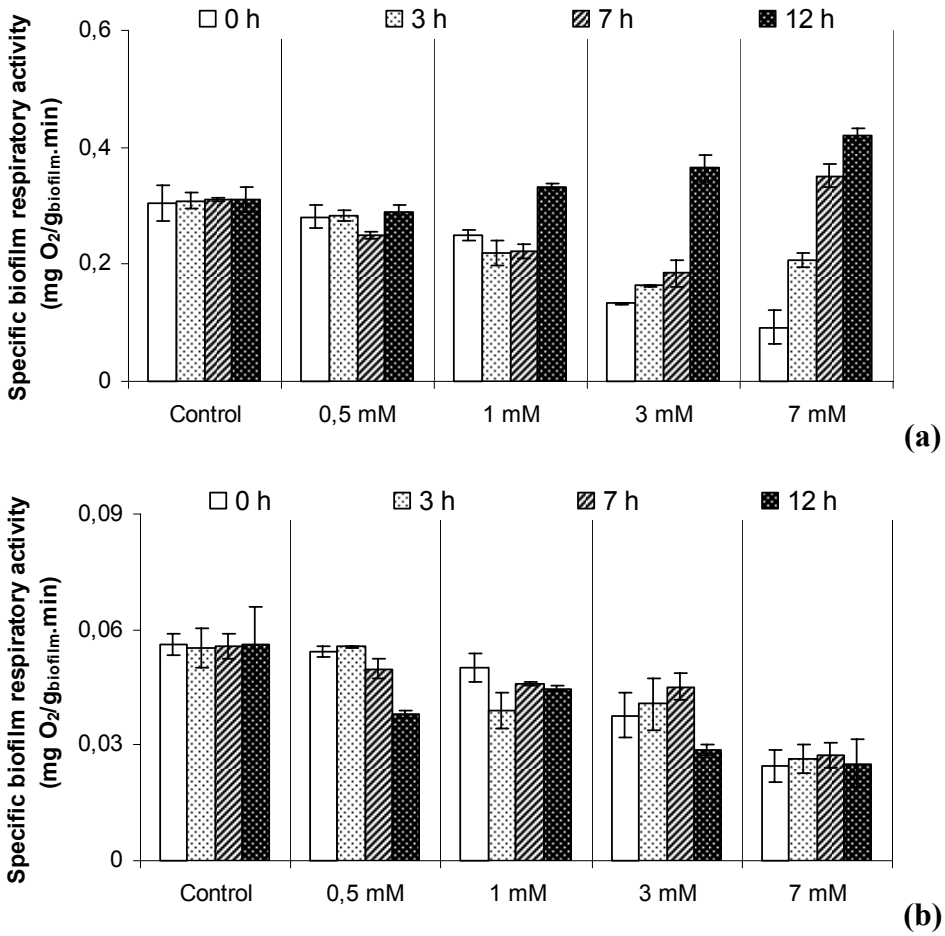


Figure 8.8 Biofilm respiratory activity after chemical treatment (0 h) with SDS and 3, 7 and 12 h later for biofilms formed under turbulent (**a**) and laminar (**b**) flow. Control means without surfactant treatment.

Concerning the behaviour of biofilms after SDS application (Figure 8.8), the activity of biofilms increased with time, particularly when 3 mM and 7 mM of SDS were applied to the biofilms. However, for turbulent biofilms the recovery is more pronounced than for the laminar biofilms ($P < 0.05$). Also, for turbulent biofilms, after SDS application, the recovery is more pronounced with the increase of the SDS concentration applied to the biofilms.

Comparing the results of biofilm recovery after CTAB and SDS application, the recovery is more evident for both biofilms treated with CTAB, being less clear for laminar

biofilms when treated with SDS (Figure 8.8). The ionic nature of the surfactant seems to be responsible for the alteration effects of the biofilm respiratory activity, playing a more significant action when the surfactant concentrations applied were near the CMC. Consequently, the biofilm recovery must be associated with the stress conferred by the surfactant application. Probably, the surfactant may have increased the availability of nutrients to the cells embedded in the biofilms (promoting bacterial recovery) since the surfactant may have changed the structure of the biofilm matrix (as can be seen by the SEM results), namely the porosity of the biofilm, and thus favouring nutrient diffusion inside the matrix. This effect occurred, probably, without killing the microorganisms. Another feature that could contribute to biofilm recovery was the establishment of the conditions prior to the contact with the surfactants and the supply of nutrients, being in accordance with the statement made by Chandy and Angles (2001) where they found that one of the key factors that determine bacterial recovery in drinking water distribution systems was the availability of nutrients. Also, the bacteria found within biofilms have suffered changes in the metabolic state. In same cases this metabolic state seems to be a state of higher metabolic activity than the one found for the control experiment. This preservative recovery, according to Stewart (2003) could lead to populations of resistant bacteria, which may be recalcitrant to a subsequent disinfection process. The control experiments (without surfactant application) did not show any expressive variation on respiratory activity along the time for both biofilms tested since, biofilms were in a metabolic steady-state (Pereira *et al*., 2001).

The overall results suggested that if the biofilms were left in the flow cell reactors longer, probably, the recovery of biofilm will be more evident and consistent.

Figure 8.9 shows representative microphotographs of biofilm formed under turbulent flow stained with Live/Dead *Bac*Light kit before, immediately after and 12 h after treatment with 0.5 mM of CTAB and 3 mM of SDS. These surfactant concentrations were opted, since the percentage of inactivation was similar for both experiments.

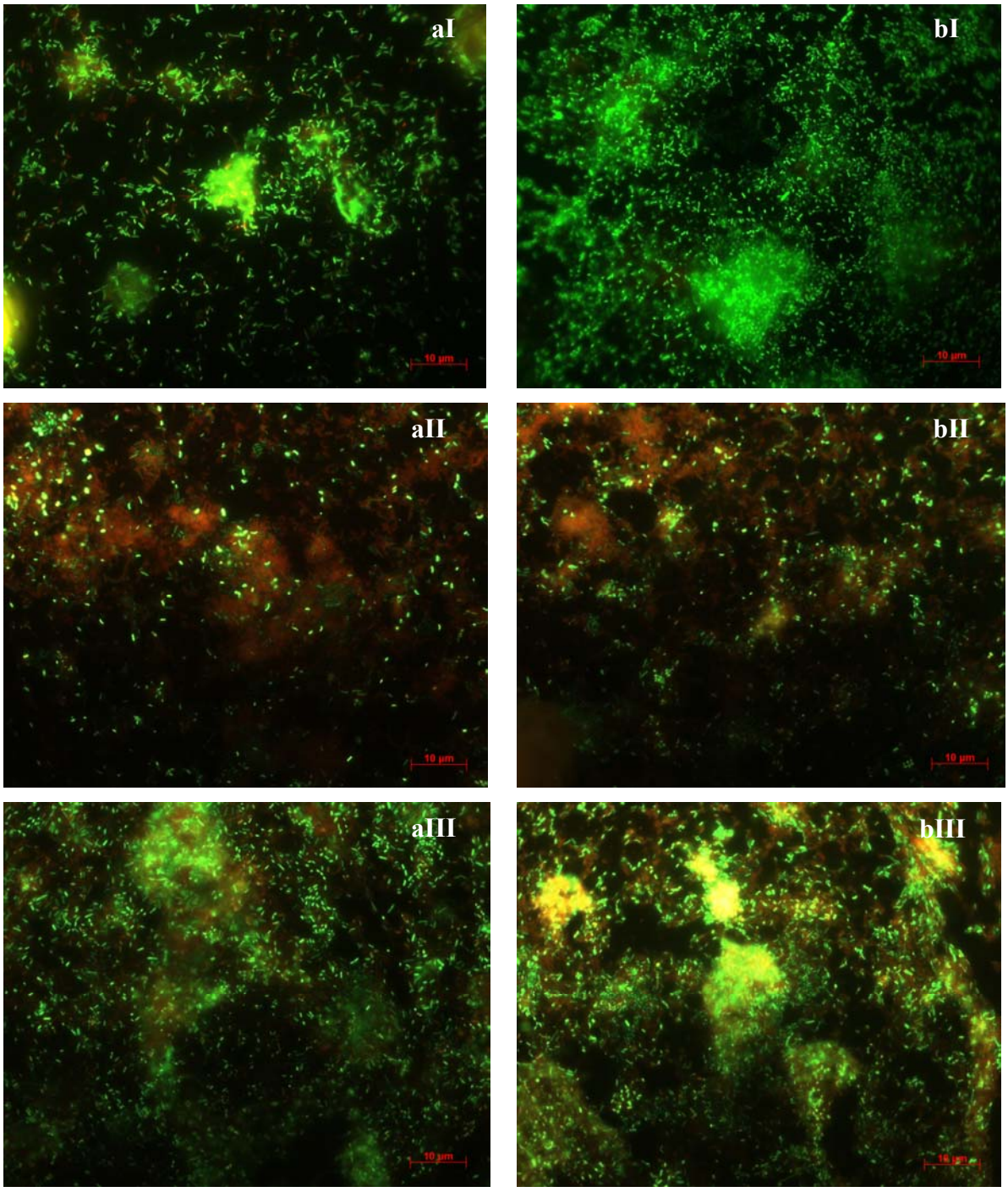


Figure 8.9 Epifluorescence photomicrographs of cells grown within biofilms formed under turbulent flow before treatment with 0.5 mM of CTAB (**aI**) and 3 mM of SDS (**bI**); immediatly after treatment (**aII**; **bII**); 12 h later (**aIII**; **bIII**). X 1320 magnification, bar=10 um.

The biofilm left on the flow cell after surfactant treatment recovered their viability during the 12 h of the experiment, corroborating the respiratory activity results (Figures 8.7 and 8.8).

The dry biofilm mass before and after surfactant application can be observed in Figures 8.10 and 8.11, respectively for CTAB and SDS.

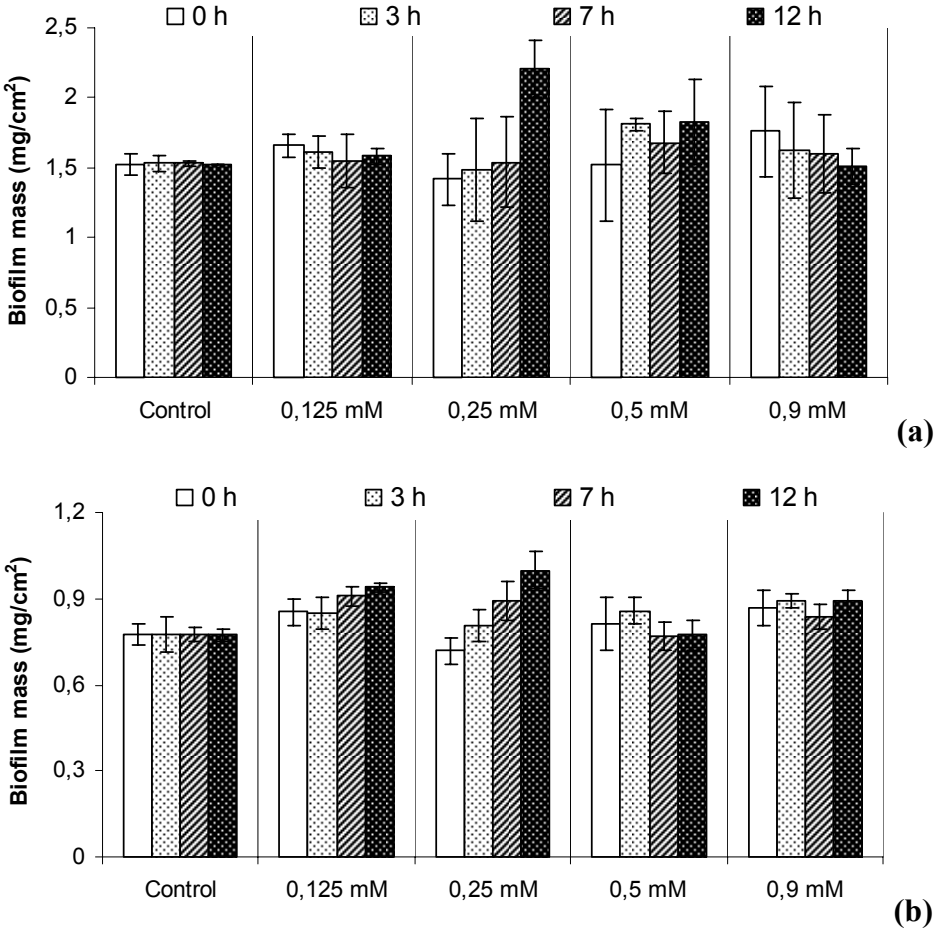


Figure 8.10 Biofilm mass after chemical treatment (0 h) with CTAB and 3, 7 and 12 h later for biofilms formed under turbulent (**a**) and laminar (**b**) flow. Control means without surfactant treatment.

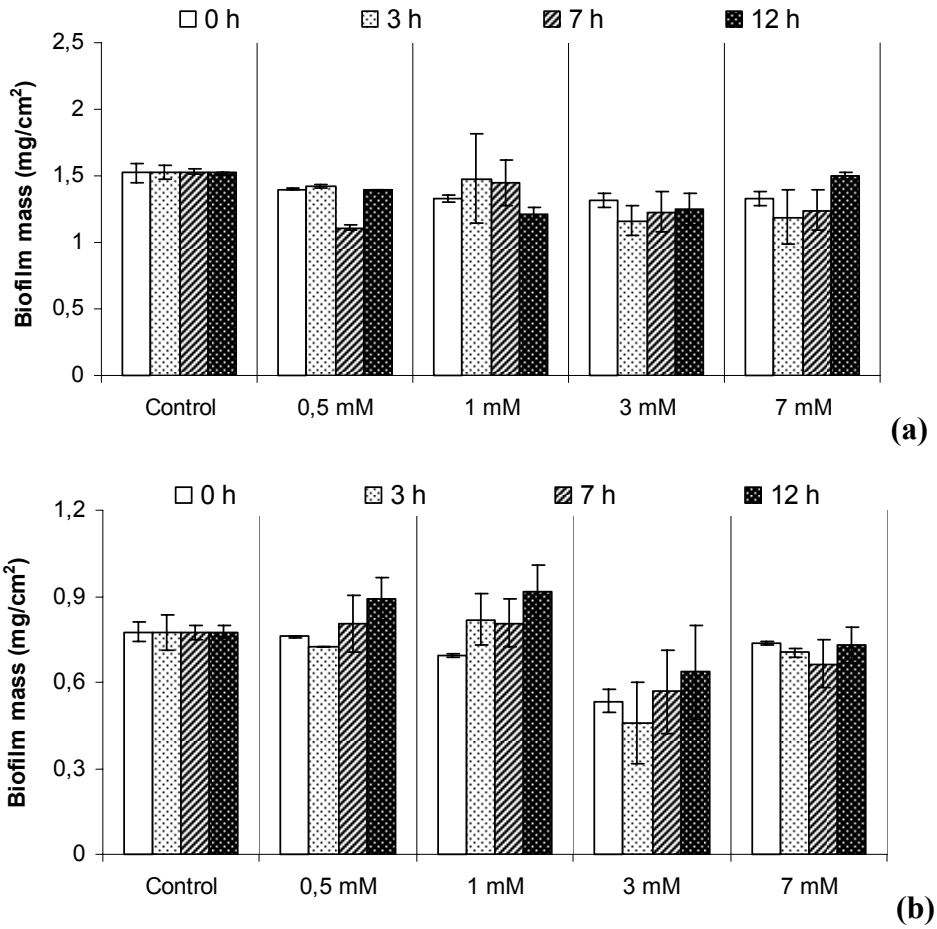


Figure 8.11 Biofilm mass after chemical treatment (0 h) with SDS and 3, 7 and 12 h later for biofilms formed under turbulent (**a**) and laminar (**b**) flow. Control means without surfactant treatment.

It can be seen that in terms of total biofilm mass, only small variations were achieved with the surfactant treatment, being those variations more noticeable for laminar biofilms treated with SDS. The application of CTAB to both turbulent and laminar biofilms did not give rise to the biomass decrease. On the contrary, it seems that the application of CTAB increased the amount of biofilm adhered to the stainless steel slides. Therefore, it is clear that the application of SDS or CTAB and the time did not promoted any significant additional biofilm removal or growth, for any conditions tested and for any sampling time ($P > 0.05$ – for both surfactants and for every condition tested).

Data presented in this study proved that the surfactant did not induced suppression of biofilm recovery in terms of biofilms activity and did not promoted the gradual biofilm erosion for both biofilms.

8.4 CONCLUSIONS

A better understanding of biofilm response face to an external stress condition is essential for the emerge of new strategies for controlling biofilms. Biofilms formed under laminar flow were more susceptibly to the inactivation effect than turbulent biofilms, but none of them were removed by the surfactants tested. A post-surfactant effect was noticed for both biofilms since they recovered their metabolic activity along time, after surfactant treatment. The application of CTAB and SDS induced biofilm recovery in terms of biofilm respiratory activity. Concerning biofilm mass, the surfactants did not promote a slow biofilm detachment or the increase in the biofilm mass, probably due to the limited time of experiment. The permanence of this remaining pellicle that is still active, or in another metabolic state, may be a source of problems, such as biofilm recovery, development of resistant biofilms or a nutrient for other microorganisms.

Both surfactants reacted with the biofilm constituents, changing their structure.

This study reinforces the inadequacy of planktonic tests as a mean to assess the efficacy of chemical agents to be used against biofilms.

This improvement in the understanding of the relationship between surfactant molecular properties, antibacterial properties and mechanisms of action could facilitate the design of chemical mixtures that more effectively control biofilm activity and removal.

Chapter 9

The role of chemical treatment and mechanical cleaning in the control of biofilms

Abstract

In this chapter, a new experimental methodology is tested in order to ascertain the mechanical stability of biofilms, by using a bioreactor combined with a stainless steel rotating device, immersed in a bioreactor, where biofilms formed by *P. fluorescens* are allowed to grow at a defined Reynolds number of agitation. These biofilms are characterized in terms of biochemical composition, amount of mass metabolic activity, structural characteristics and mechanical stability. Afterwards, the biofilms are submitted to chemical aggression, followed by mechanical treatments by submission to increase Reynolds number of agitation promoted by the increase of the rotation speed of the stainless steel cylinders where biofilms are formed. The effect of the exposure for ½ h to: GTA, OPA, CTAB, SDS, NaOH and SHC is assessed in order to ascertain the effect of chemical agents on the biofilm mechanical stability. These synergistic chemical and mechanical treatments are also evaluated as a means to control biofilms.

9.1 Introduction

Bacterial biofilms associated with surfaces are complex three-dimensional structures in which bacteria are embedded in a matrix chiefly made of EPS (Campanac *et al.* 2002). A better understanding of biofilm behaviour is particularly important due to the many serious problems associated with their presence (Pereira and Vieira, 2001). The EPS matrix provides the biofilm mechanical stability by filling and forming the space between the bacterial cells, keeping them together (Körstgens *et al.* 2001). Once developed, biofilms are harder to be removed completely (Pereira and Vieira, 2001). Chemical agents and mechanical forces are parameters often involved simultaneously in the control of biofilms, since the application of sole chemical agents tends to leave the biofilm intact when no mechanical treatment is implemented in the control process (Flemming, 1996). Mechanical stability is an important factor in determining the structure and function of biofilm systems and this parameter plays a key role in the removal and/or control of biofilms in engineered systems (Poppele and Hozalski, 2003). So far, very limited studies have been done regarding the mechanical stability of biofilms (Ohashi and Harada, 1994; 1996; Ohashi *et al.* 1999; Stoodley *et al.* 1999; Körstgens *et al.* 2001; Poppele and Hozalski, 2003). Moreover, studies concerning the effect of chemical agents on the biofilm mechanical stability are even fewer.

In this chapter, a bioreactor system that allows the formation and subsequent exposure of biofilms to different chemical and mechanical stresses is used to assess the synergistic action of chemical and mechanical treatment on biofilm removal and to characterize the intrinsic biofilm mechanical stability.

9.2 Materials and methods

9.2.1 Microorganism and culture conditions

The microorganism used was *P. fluorescens* ATCC 13525^{T}. The culture conditions were followed according to the procedure described in sub-chapter 3.1.2.

9.2.2 Biofilm formation

Biofilms were grown on stainless steel (ASI 316) cylinders, according to the procedure described in sub-chapter 3.4.

9.2.3 Mechanical stability of the biofilm

After 7 d of biofilm formation, the cylinders *plus* biofilm were carefully removed from the 3.5 l reactor. One of the cylinders was then immersed in a reactor with phosphate buffer, (control experiment), while the others were immersed in reactors containing different chemical solutions (volume of each reactor was 170 ml). This chemical treatment was carried out with the cylinders rotating at 300 min^{-1} during ½ h. Afterwards, the cylinders were removed from the reactors containing the chemical solutions, accurately weighed, introduced in other reactors with phosphate buffer and consecutively subjected to serial velocities of rotation, *i.e.*, 500, 1000, 1500, and 2000 min^{-1}, for a period of 30 s each. The wet weight of the cylinders *plus* biofilm attached was determined before and after each rotation. The experiments were repeated in three different occasions for every chemical treatment tested.

For each experiment, the stainless steel cylinders were identified and weighed before being introduced in the reactor. The same procedure was followed with the control assay, *i.e.*, with the cylinder *plus* biofilm immersed in the buffer solution.

The wet mass of the biofilm that was removed from the surface area of each cylinder, after each rotation speed, was expressed in percentage of biofilm removal, and the amount of biofilm that remained adhered after submission to the completely series of rotation speed was expressed as percentage of biofilm remaining, according to the following equations:

$$Biofilm\ remaining\ (\%) = [(X_{2000} - X_c)/(X_{after\ treat} - X_c)] \times 100 \quad (9.1)$$

$$Biofilm\ removal_{\ 500\ min^{-1}}\ (\%) = [(X_{after\ treat} - X_{500})/(X_{after\ treat} - X_c)] \times 100 \quad (9.2)$$

$$Biofilm\ removal_{\ 1000\ min^{-1}}\ (\%) = [(X_{500} - X_{1000})/(X_{after\ treat} - X_c)] \times 100 \quad (9.3)$$

$$Biofilm\ removal_{\ 1500\ min^{-1}}\ (\%) = [(X_{1000} - X_{1500})/(X_{after\ treat} - X_c)] \times 100 \quad (9.4)$$

$$Biofilm\ removal_{\ 2000\ min^{-1}}\ (\%) = [(X_{1500} - X_{2000})/(X_{after\ treat} - X_c)] \times 100 \quad (9.5)$$

$X_{after\ treat}$ – Wet biofilm *plus* cylinder after the treatment during ½ h.

X_c – wet mass of the cylinder.

X_{500}, X_{1000}, X_{1500}, X_{2000} – wet mass of the biofilm plus cylinder after submission to respectively 500, 1000, 1500 and 2000 min^{-1}.

Assuming the system with a behaviour of an agitated vessel, the Reynolds number for each rotation speed can be calculated according to the following equation (Geankoplis, 1993):

$$N`Re_A = \frac{Da^2 \times N \times \rho}{\mu} \qquad (9.6)$$

Where, Da (m) is the diameter of the cylinder; N (rotation/s) is the rotation speed; ρ (Kg/m^3) is the fluid density; μ (Kg/m.s) is the fluid viscosity.

Table 9.1 Reynolds number of agitation for each rotation speed used in this study

min^{-1}	$N`Re_A$
300	2400
500	4000
1000	8100
1500	12100
2000	16100

9.2.4 Chemicals used to treat the biofilms

In the present work, the follow chemical agents were used:

Two non-oxidizing aldehyde-based biocides:

OPA at 50, 100, 200 and 300 mg/l, and GTA at 100, 200, 500 and 1000 mg/l.

Two surfactants:

CTAB at 0.125, 0.250, 0.500 and 0.900 mM, and SDS at 0.5, 1, 3 and 7 mM.

Two oxidizing biocides:

NaOH at 50, 200, 300 and 500 mM, and SHC at 50, 200, 300 and 500 mg/l.

9.2.5 Biofilm characterization

The biofilm that covered the stainless steel cylinder was completely scraped off the metal and resuspended into 10 ml of phosphate buffer pH 7. This biofilm suspension was used to assess the cellular respiratory activity of the biofilm through oxygen uptake rates and afterwards biofilm mass. Biofilm from an other cylinder was resuspended in extraction buffer for further quantification of its extracellular and cellular proteins and polysaccharides content.

The experiments were repeated in three different occasions by performing three independent biofilm formation experiments and biofilm characterization was only carried out with biofilms that were not treated with chemical agents.

9.2.6 Respiratory activity

The respiratory activity of the biofilm was evaluated by measuring oxygen uptake rates due to glucose consumption in a biological oxygen monitor (BOM) in short-term assays, according to the procedure described in sub-chapter 3.6.6.

9.2.7 Extracellular polymeric substances extraction procedure

Extraction of the EPS of the biofilm was carried out according to the procedure described in sub-chapter 3.6.2.

9.2.8 Biochemical analysis

The biochemical analyses were carried out with the homogenised biofilm suspensions. The amount of total, extracellular and cellular proteins and polysaccharides were determined according to the procedure described in sub-chapter 3.6.3.

9.2.9 Biofilm mass

The wet biofilm mass was assessed by the difference between the cylinder *plus* biofilm before the treatment and the clean cylinder.

The dry biofilm mass was assessed by the determination of the TVS of the homogenised biofilm suspensions, according to the procedure described in sub-chapter 3.6.5.

9.2.10 Statistical analysis

Wilcoxon procedure was used to compare the equivalence between the different $N`Re_A$ for the same chemical concentration and for the same $N`Re_A$ for the different chemical concentrations.

9.3 Results and Discussion

9.3.1 Characterization of the biofilms formed on the rotating device

Figure 9.1 shows a stainless steel cylinder covered with biofilm after 7 d of growth.

Figure 9.1 Stainless steel cylinder covered with biofilm after 7 d of growth.

This Figure clearly shows that the surface of the stainless steel cylinder was completely covered with a thick and slimy biofilm that seems to be strongly adhered to the surface. Some characteristics of the biofilms formed on the cylinders of the rotating device, namely the biofilm activity, mass, proteins and polysaccharides content, are presented in Table 9.2. This characterization was performed with biofilms before the submission to the chemical and mechanical treatments.

Table 9.2 Characteristics of the biofilms formed on the surface of the stainless steel cylinders after 7 d of growth

Biofilm activity ($mg\ O_2/g_{biofilm.min}$)	0.150 ± 0.02	
Biofilm mass (mg/cm^2)	Dry	0.907 ± 0.01
	Wet	21.5 ± 6.1
Proteins ($mg/g_{biofilm}$)	Total	210 ± 19
	Matrix	59.9 ± 15
	Cells	150 ± 17
Polysaccharides ($mg/g_{biofilm}$)	Total	200 ± 4.6
	Matrix	121 ± 56
	Cells	79 ± 18

From Table 9.2 it can be verified that the biofilms were metabolically active, since it showed the ability to oxidize glucose (Chapter 3), and contained about 96 % of water, which is in accordance with other authors (Vieira *et al.,* 1993; Azeredo and Oliveira, 2000; Pereira *et al.,* 2001a). The amount of extracellular proteins was about 29 % of the total biofilm proteins and the amount of extracellular polysaccharides was nearly 62 % of the total biofilm polysaccharides. The total protein content was similar to the total polysaccharide content, besides the analytical methods used were different; consequently, comparison between total proteins and polysaccharides can not be feasible.

The characteristics of the biofilms formed on the stainless steel cylinders (Table 9.2), namely the respiratory activity, biofilm mass and total content of proteins and polysaccharides, are similar to the ones observed in biofilms formed in the flow cell system under turbulent flow (Chapter 5), specifically the significant content of extracellular proteins and polysaccharides found in the composition of the biofilm matrix. The evidence of the slimy matrix of the biofilm depicted in Figure 9.1 acquired great importance in biofilm architecture, and thus in biofilm mechanical stability, since, according to Körstgens *et al.* (2001), EPS are responsible for keeping biofilm together and binding the biofilm to the support, forming a temporary network of fluctuating junction points.

9.3.2 Biofilm removal due to mechanical stress

Figure 9.2 shows the biofilm removal obtained due to the increase in the $N`Re_A$ for the control experiment.

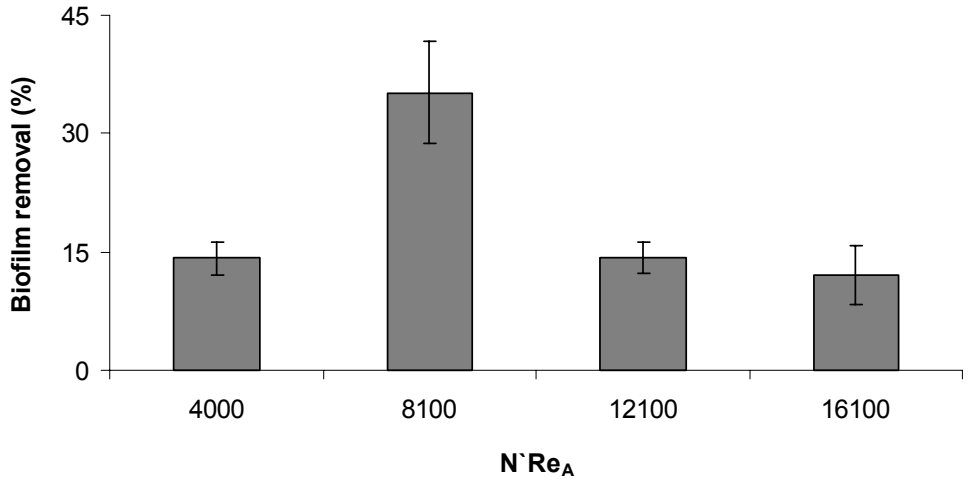


Figure 9.2 Biofilm removal for the control assay due to change in the $N`Re_A$.

The existence of shear stress forces higher than the one under which the biofilm was formed ($N`Re_A$ = 2400) caused biofilm removal. The high percentage of removal occurred with the implementation of a rotation of velocity that corresponds to a Reynolds number of 8100 (Figure 9.2), being biofilm removal similar for the others Reynolds number tested. So, it can be said that the biofilm removal is dependent on the hydrodynamic conditions ($P < 0.05$). Figure 9.2 also shows that the total series of Reynolds number did not give rise to total biofilm removal, since only about 76 % of biofilm mass was detached from the cylinders.

The mechanical stability of biofilms, *i.e.,* the behaviour of biofilms face to external stress mechanical conditions, is of great impact for both wanted and unwanted biofilms (Poppele and Hozalski, 2003). In this study, the mechanical stability of the biofilm was assessed by submitting biofilms to different shear stress, correspondent to increasing $N`Re_A$, which may weak the biofilm structure and promote detachment. The biofilm formed on the cylinders of the rotating device prior to chemical stress was characterized in order to determine the inherent biofilm mechanical stability, since detachment processes may be dependent on it. According to Stoodley *et al.* (1999a), biofilm matrix develops an inherent internal tension, which is in equilibrium with the shear stress under which the biofilm is

formed. The EPS strength the cohesive forces within the biofilm, thereby contributing to an enhanced inherent biofilm mechanical stability (Azeredo and Oliveira, 2000). The EPS can mediate both cohesion and adhesion of cells, and play a crucial role in maintaining structural integrity of the biofilm matrix (Liu and Tay, 2001). The removal of a well established biofilm requires the overcome of the forces which maintain the integrity of the biofilm (Körstgens *et al.* 2001). This control experiment (Figure 9.2) showed that biofilms subjected to sole mechanical treatment were hardly removed with low shear stress ($N`Re_A \leq 4000$) since only about 14 % of biofilm removal was achieved. However, when the $N`Re_A$ were raised from 4000 to 8100 a noticeable biofilm detachment was observed, but a layer remained on the surface even when the highest $N`Re_A$ was applied. According to Azeredo and Oliveira (2000), the biofilm detachment is processed in layers, where the increase in the shear stress may progressively thin the biofilm, being mechanical failure and total detachment the ultimate effect expected. This removal of biofilms from surfaces using increasing shear stress promoted by the increasing in the $N`Re_A$ is a mechanical phenomenon. However, the most common practice to eliminate unwanted biofilms involves the application of toxic chemicals (Chen and Stewart, 2000).

9.3.3 Biofilm removal due to mechanical stress after exposure to non-oxidizing biocides

Figures 9.3 and 9.4 show the biofilm removal caused by the implementation of the different Reynolds number after biofilm be treated with OPA and GTA, at different concentrations.

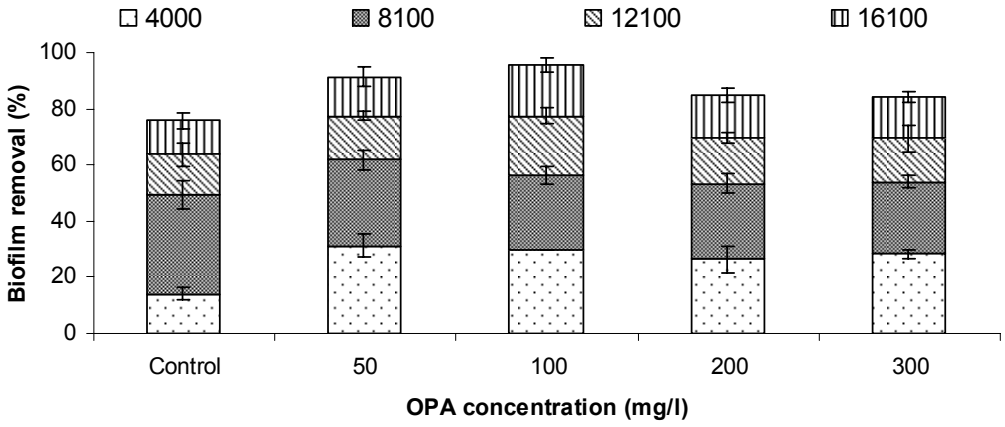


Figure 9.3 Biofilm removal observed after the alteration of the $N`Re_A$ for the biofilm control and for the OPA treated biofilms. Control means without OPA treatment.

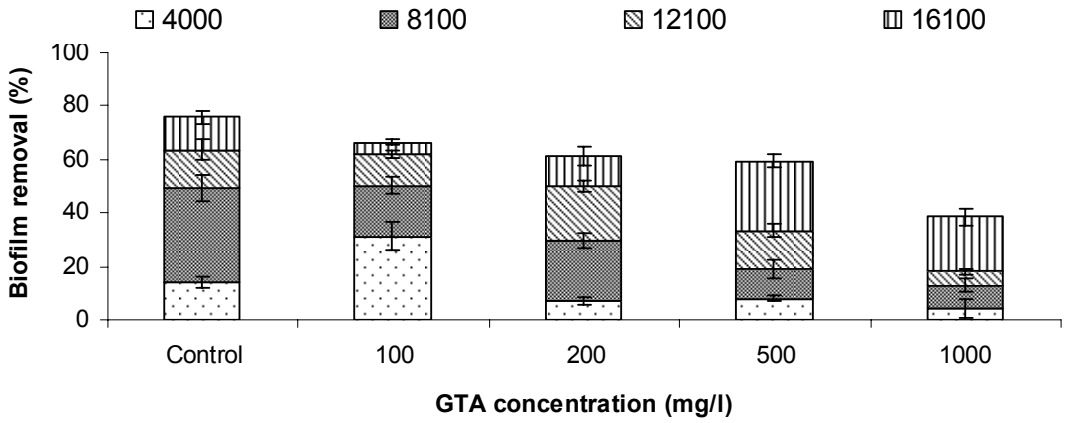


Figure 9.4 Biofilm removal observed after the alteration of the N`Re_A for the biofilm control and for the GTA treated biofilms. Control means without GTA treatment.

The results obtained with OPA show that its application to biofilms to favour the detachment caused by the change in the N`$Re_A$. The increase in this biocide concentration does not have significant effect in the biofilm removal, since, for the same N`Re_A (Figure 9.3) the biofilm removal was similar for every concentration tested ($P > 0.5$). It also can be noticed that the percentage of biofilm removal with the lower N`$Re_A$ applied increased when biofilms were previously treated with OPA. The comparison between the different N`Re_A for the same OPA concentration shows that, only for 50 mg/l of OPA the biofilm removal exhibited significant differences ($P < 0.05$).

Figure 9.4 shows that the total biofilm removal achieved with the total series of Reynolds number decreased with the increase of the GTA concentration used to previously treat biofilms. Moreover, for the lower N`Re_A applied biofilm removal decreased with the increase in the GTA concentration. These facts suggest that the biofilm previously treated with GTA becomes less susceptible to the alteration of the shear forces. For each GTA concentration tested, the statistical analysis of the biofilm removal values achieved after each N`$Re_A$ showed that they were not equivalent ($P < 0.05$). The comparison between the different GTA concentrations tested, for the same N`Re_A, shows that biofilm removal are significantly different ($P < 0.05$). This result shows that GTA application and mechanical treatment had a significant effect on the variation of biofilm removal.

GTA was not efficient at removing the biofilm from the stainless steel cylinders in spite of the fact that this biocide is frequently used to chemically control the accumulation of biofilms (Pereira and Vieira, 2001). On the contrary, GTA contributed to the formation of a harder deposit, since the percentage of biofilm remaining on the surface was higher than

for the control experiment. The bi-functional nature of GTA allows it to react and cross-link with ammonia and primary amine groups and more slowly with secondary amines (Walsh *et al.* 1999b; McDonnell and Russell, 1999). Following the hypothesis of GTA as a cross-linking agent would lead to predict that biofilm treatment with GTA should actually stabilize the biofilm, as found with this work. Conversely, the results obtained with OPA are consistent with its less effect of cross-linking when compared with GTA. Probably this fact is related with the aromatic ring presented in the molecular structure of OPA which confers a diminished flexibility of the molecule, conversely to the aliphatic chain of GTA (Walsh *et al.* 1999a; 1999b; Simons *et al.* 2001). Consequently, the biofilm remaining on the surface decreased slightly after OPA application in relation to the control.

9.3.4 Biofilm removal due to mechanical stress after exposure to surfactants

Figures 9.5 and 9.6 shows, biofilm removal caused by the exposure of the biofilm to the different Reynolds number after treatment with respectively CTAB and SDS, at different concentrations.

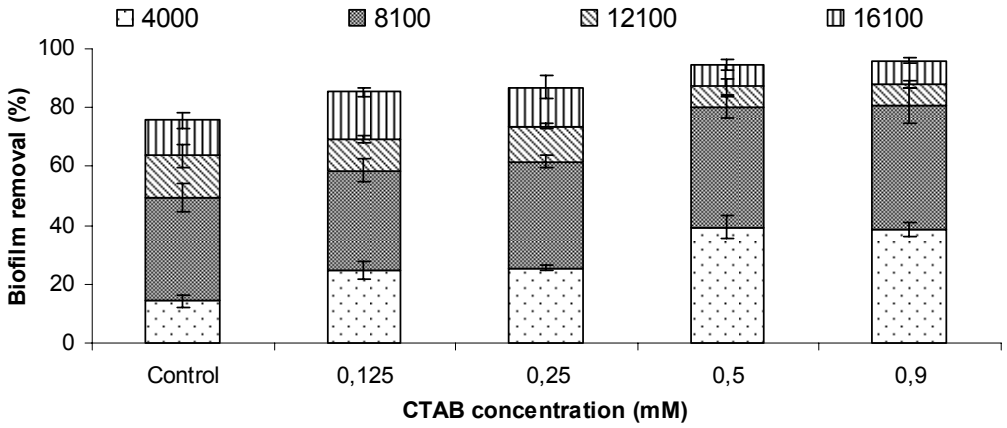


Figure 9.5 Biofilm removal observed after the alteration of the N`Re_A for the biofilm control and for the CTAB treated biofilms. Control means without CTAB treatment.

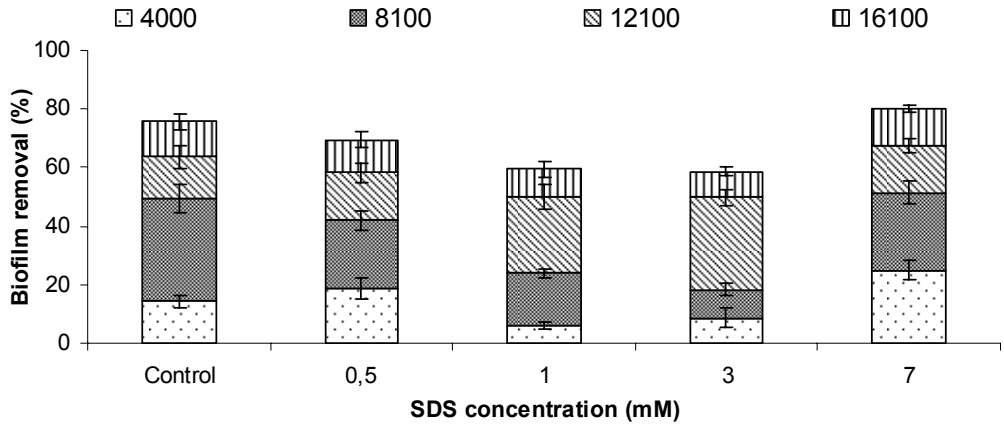


Figure 9.6 Biofilm removal observed after the alteration of the N`Re_A for the biofilm control and for the SDS treated biofilms. Control means without SDS treatment.

CTAB enhances biofilm removal in respect to the control, and increases with CTAB concentration. The results also show that, the biofilm removal happens in a higher extent for the smaller (4000 and 8100) N`$Re_A$ (Figure 9.5). This trend becomes more important with the increase of CTAB concentration (Figure 9.5). For the same CTAB concentration the biofilm removal values observed for the different N`Re_A are statistically different (P < 0.01), showing that CTAB application increases the biofilm susceptibility to detachment through the mechanical action. However, when comparing the biofilm removal within concentrations and for the same N`Re_A, only for 4000 the biofilm removal was significantly different (P < 0.05).

Concerning SDS, apart from 7 mM, its application to the biofilm resulted in the decrease of biofilm removal achieved with the hydrodynamic change. Conversely, with the application of 7 mM of SDS biofilm removal happened in a higher extent for N`$Re_A$ of 4000 and 8100, but, similar with the others N`Re_A tested (P > 0.05). For 0.500 mM the biofilm removal is similar for every N`$Re_A$ tested ($P$ > 0.1). The application of 1 and 3 mM of SDS promoted significant differences in the posterior biofilm removal ($P$ < 0.05), when comparing the different N`Re_A, being the high amount of biofilm removal promoted with the exposure to a N`$Re_A$ of 12100. However, when comparing the biofilm removal for the same N`Re_A within the different concentrations, only for a N`Re_A of 4000 was found a significant difference (P < 0.05) due to the high amount of biofilm removal found after treatments with 0.5 and 7 mM. Accordingly, the treatment with surfactants caused different biofilm responses that may be related with their chemical nature. Concerning cationic surfactants, their action is attributed to their positive charge that forms an electrostatic bond

with negatively charged sites (Cloete *et al.* 1998). The increase in the CTAB concentration promoted the subsequent higher biofilm removal due to the destabilization of the biofilm cohesive forces, being biofilm removal detected at a higher extent to the smaller shear stresses (Figure 9.5). The effect of SDS on the mechanical stability of the biofilm may be due to the disruption of the hydrophobic interactions involved in cross-linking the biofilm matrix (Chen and Stewart, 2000). However, in this work, this SDS effect was only felt for the higher concentration (7 mM) tested, proposing that low concentrations of SDS can even promote the strength of the biofilm structure. The different biofilm behaviour may be related with the chemical reaction of the surfactants with the biofilm components used, that can give rise to the strengthening or to the weakening of the biofilm structure. The electrostatic bonds created stress or cross-linking depending on the chemical structure of the molecule.

9.3.5 Biofilm removal due to mechanical stress after exposure to oxidizing biocides

Figures 9.7 and 9.8 show biofilm removal caused by the exposure of the biofilm to the different N`Re_A after treatment with NaOH and SHC at different concentrations.

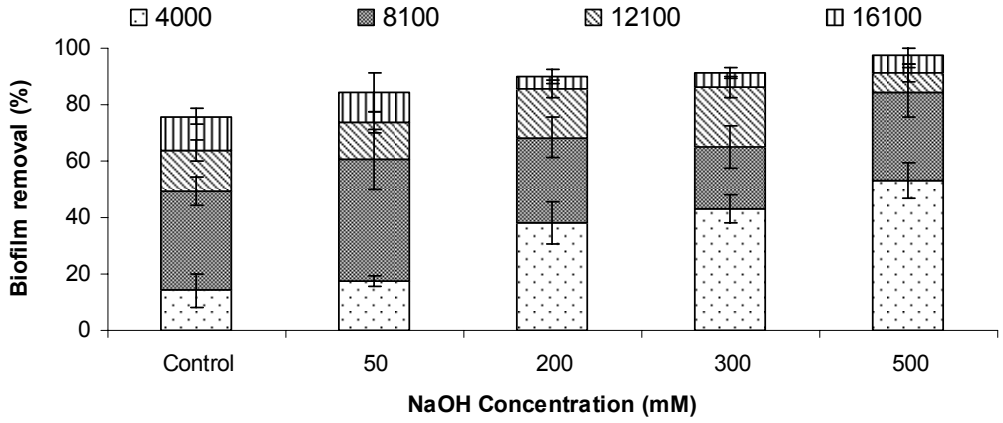


Figure 9.7 Biofilm removal observed after the alteration of the N`Re_A for the biofilm control and for the NaOH treated biofilms. Control means without NaOH treatment.

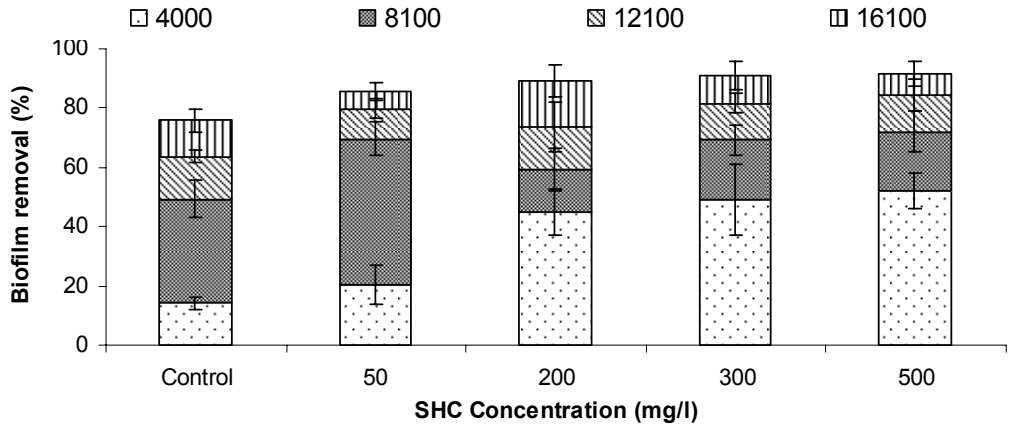


Figure 9.8 Biofilm removal observed after the alteration of the N`Re_A for the biofilm control and for the SHC treated biofilms. Control means without SHC treatment.

Similar impacts on biofilm removal were found for NaOH and SHC (Figures 9.7 and 9.8). Both chemicals affected similarly biofilm removal for every condition tested. Concerning NaOH, with the exception for 50 mM, the highest amount of biofilm removal is found for a N`$Re_A$ of 4000 and with the trend to increase with the increase in the concentration applied. For 50 mM the high amount of biofilm removal was found with the exposure to an exposure to a N`Re_A of 8100. However, the biofilm removal is statistically equivalent when comparing with the others N`$Re_A$ ($P < 0.05$). Concerning the comparison of the different N`Re_A for the same NaOH concentration, the results are significantly different ($P < 0.05$), with the exception for the treatment with 200 mM ($P > 0.10$) where the biofilm removal happened in a similar extent with the submission to a N`Re_A of 4000 and 8100.

The application of 50 mg/l of SHC resulted in a posterior biofilm removal that reached the highest amount with the exposure to a N`$Re_A$ of 8100. For the others concentrations tested, the biofilm removal was high for a N`Re_A of 4000. The biofilm removal was similar for every concentration tested when comparing for the same N`$Re_A$ ($P < 0.05$). The results were significantly different ($P < 0.05$) as a consequence of the higher biofilm removal with the increase of N`Re_A.

According to Kim *et al.* (2002), oxidizing agents are widely used as disinfectants since they cause deleterious effects on bacteria, affecting the bacterial respiratory and transport activities, and nucleic acids. In this study, the oxidizing biocides, probably, reacted strongly with the EPS matrix, destroying the structure which became more vulnerable to

hydrodynamic stress. So, it is not surprising to obtain more removal for the same N`Re_A as the concentration increase.

9.3.6 Total biofilm remaining on the surface

The total percentage of biofilm that was not removed in the control experiment and for the experiments with the application of the different chemicals prior to the submission to the total series of N`Re_A, considered as the biofilm remaining, is presented in Table 9.3.

Table 9.3 Total percentage of biofilm remaining on the surface for the several chemical treatments and for the control experiment after the submission to the total series of N`Re_A

	Treatment	Total biofilm remaining (%)
	Control (without chemical treatment)	24.2 ± 0.59
	OPA	
Non-oxidizing biocide	50 mg/l	15.6 ± 4.3
	100 mg/l	14.3 ± 3.3
	200 mg/l	14.8 ± 5.6
	300 mg/l	15.0 ± 3.1
	GTA	
Non-oxidizing biocide	100 mg/l	33.8 ± 3.3
	200 mg/l	35.4 ± 9.9
	500 mg/l	40.6 ± 2.1
	1000 mg/l	61.5 ± 1.8
	CTAB	
Cationic surfactant	0.125 mM	14.8 ± 1.3
	0.250 mM	13.2 ± 2.8
	0.500 mM	5.31 ± 0.72
	0.900 mM	4.16 ± 0.35
	SDS	
Anionic surfactant	0.500 mM	30.6 ± 4.5
	1.00 mM	40.7 ± 4.2
	3.00 mM	41.6 ± 6.2
	7.00 mM	19.7 ± 3.9
	NaOH	
Oxidizing biocide	50 mM	15.7 ± 4.8
	200 mM	10.1 ± 3.9
	300 mM	8.63 ± 1.7
	500 mM	2.89 ± 2.1
	SHC	
Oxidizing biocide	50 mM	14.1 ± 5.4
	200 mM	10.8 ± 4.9
	300 mM	8.95 ± 5.6
	500 mM	8.48 ± 1.9

From this Table, it is possible to emphasize that for the control assay, the biofilm remaining, after submission to the total series of $N`Re_A$ was about 24%. The addition of several chemicals to biofilms lead to different percentages of biofilm remaining that ranged from 3 % to 62 %. Treatments that promoted a similar or higher percentage of biofilm remaining than for the control assay were GTA for every condition tested and SDS at 0.5, 1 and 3 mM. Same range of values of biofilm percentage remaining on the surface as the control assay were the experiments with 0.125 mM of BC and 7 mM of SDS.

After the chemical treatment of the biofilm, their EPS matrix often remained more or less unaffected and thus biofilm was left in place (the amount of biofilm removed due to the exposure to the chemical agents during ½ h was about 5 ± 2 % for every condition tested). This biofilm can act as an additional source of nutrients and/or as a suitable surface to further growth of cells. So, in this work, together with shear forces variation (through the increase in the $N`Re_A$) the coupled action of a set of chemicals in biofilm mechanical stability was applied in order to obtain a clean surface, since besides the chemical agents could interact with the cohesive forces of the biofilm, causing the destabilization of the structure, the synergistic action of chemical and mechanical treatment seems to be the main strategy for biofilm control.

9.4 Conclusions

The system presented in this work provided an approach to investigate the influence of several parameters on the mechanical stability of biofilms, leading to a better understanding of biofilms in different environments and the development of biofilm control strategies.

From the overall results, it can be concluded that the effect of the chemical compounds on the biofilm removal and consequent biofilm mechanical stability varied with the chemical nature and that chemical treatment is far from being a factor that induces massive detachment. Even with the synergistic chemical and mechanical treatment total biofilm eradication was not achieved in this work for every condition studied.

The characterization of the biofilm showed that the system tested allowed to the formation of a great amount of biofilm that covered the surface of the stainless steel cylinder, being this biofilm metabolic active, vastly constituted of extracellular substances and having an inherent mechanical stability.

The application of OPA to the biofilms favoured the detachment caused by the increase in the mechanical stress, being the biofilm removal not dependent with the increase of the OPA concentration. Also, OPA showed to be an alternative to GTA as a means to control unwanted biofilms. The biofilms treated with GTA showed posterior recalcitrance properties when exposed to mechanical stress conditions, being this effect more pronounced with the increase in the GTA concentration.

The treatment of biofilms with CTAB caused the posterior biofilm removal that was more pronounced with the increase of the concentration applied and with the increase on the mechanical stress conditions. The application of SDS, only for the highest concentration tested, it was detected biofilm removal due to the exposure to increasing shear forces, when comparing with the control experiment. Moreover, for the smallest concentrations were observed an effect similar to the one found with GTA.

The previous application of oxidizing agents (NaOH and SHC) improved biofilm removal by mechanical action, being the effect more pronounced with the increase in their concentration.

The chemical diversity of agents tested emphasizes that are multiple interactive forces that contribute to biofilm mechanical stability. Therefore, care must be taken and the experiments must be made concerning the choice of the correct protocol for biofilm control, since several chemical compounds promoted the increase in the biofilm mechanical stability or did not have a significant effect on the weakening of the biofilm structure and the desired biofilm control.

Chapter 10

Comparison of biofilm formation by two *Pseudomonas fluorescens* strains isolated from an industrial process – an introduction to mixed biofilms

Abstract

Two *P. fluorescens* strains, isolated from a dairy processing plant with significant differences in their ribotype and enzymatic characteristics were characterized in terms of biofilm formation ability as single and mixed communities. Biofilms were allowed to growth for 7 d using the flow cell reactor, operating under turbulent and laminar flow, and the bioreactor rotating system.

Experiments with cells in planktonic state were also carried out to assess the growth patterns in order to deduce their connected behaviour in a mixed system. The OMP expression was assessed for later comparison with the ones obtained from biofilm cells.

The biofilms formed under turbulent and laminar conditions in the flow cell system were compared in terms of mass formation ability, biochemical composition, metabolic activity and structural characteristics.

The biofilms formed using the rotating system were characterized in terms of mass formation ability, biochemical composition, metabolic activity, structural characteristics and their behaviour when exposed to external mechanical stress conditions.

10.1 Introduction

Contrasting with the main laboratory researches developed nowadays (using often single microorganisms with well known characteristics), in nature, most bacteria do not exist as pure cultures. The importance of using simple laboratory tests is supported by the fact that strains isolated from real industrial processes present, generally, differential characteristics from the other strains of the same specie (Sidhu *et al.,* 2001; Langsrud *et al.,* 2003). In fact, significant proportions of all microorganisms can be associated in complex multi-species biofilms, performing community level processes (Møller *et al.,* 1998). According to Fux *et al.* (2005b), growth within mixed communities in complex environments of the real world, contrasts from the growth obtained with the standardized and idealized laboratory conditions. Lindsay *et al.* (2002) found that co-cultured bacteria in biofilms influence each other with respect to attachment capabilities and the posterior disinfection resistance/susceptibility. Additionally, interactions in a mixed community enhanced each other's survival of disinfection treatments compared with the corresponding single species biofilms (Lindsay *et al.,* 2002). So, these features play a significant role in a disinfection context (Langsrud *et al.,* 2003).

Since it appears that no bacterial strain can truly represent its specie (Fux *et al.,* 2005b), in the present chapter a comparison between two *P. fluorescens* strains isolated from an industrial process was carried out as single biofilms and when associated as mixed populations.

10.2 Materials and methods

10.2.1 *Microorganism and culture conditions*

The microorganisms used were *P. fluorescens* D3-348 and *P. fluorescens* D3-350. The culture conditions were followed according to the procedure described in sub-chapter 3.1.2.

10.2.2 Growth curves

Several flasks containing 200 ml of sterile growth medium were inoculated with an overnight bacterial suspension of each microorganism, enough to have an optical density recorded at 640 nm - $OD_{(640\ nm)}$ of 0.200 (~ 5×10^8 cells/ml), putt in an orbital shaker (120 min^{-1}, 27 ºC) and left to grow. The bacterial growth was followed by taking aseptically a 2.5 ml sample from each flask along time and recorded the $OD_{(640\ nm)}$.

At the end of the experiment, the cultures were streaked onto solid medium to ensure that they remained uncontaminated.

10.2.3 Biofilm system – flow cell reactor

Biofilm formation, under turbulent and laminar conditions, was achieved in the flow cell system according to the procedure described in sub-chapter 3.3. To obtain the mixed-strains biofilms, two independent 0.5 l reactors were used (one with *P. fluorescens* D3-348 and the other with *P. fluorescens* D3-350). The 3.5 l dilution reactor was simultaneously inoculated with the two strains and fed with the minimal nutrient medium at a flow rate two times higher (3.4 l/h) than the one used for biofilm formation by a single specie, in order to obtain the adequate dilution rate.

10.2.4 Biofilm system – bioreactor rotating system

To assess the biofilm mechanical stability, biofilm formation was promoted in the bioreactor rotating system, already described in sub-chapter 3.4 and in more detail in sub-chapter 9.2.3. In the case of mixed-strains biofilms the changes referred above, for the flow cell system, were also performed in this system.

10.2.5 Scrapping and disaggregation of the biofilms

The several biofilms that covered the metal slides were completely scrapped from the metal slides according to the procedure described in sub-chapter 3.6.1.

10.2.6 Respiratory activity

The respiratory activity of the several biofilm samples was evaluated by measuring oxygen uptake rates in a biological oxygen monitor (BOM) in short-term assays (sub-chapter 3.6.6).

10.2.7 Biofilm mass

The dry biofilm mass was assessed by the determination of the TVS of the homogenised biofilm suspensions, according to the procedure described in sub-chapter 3.6.5.

The wet biofilm mass was assessed by the difference between the weight of the cylinder *plus* biofilm and the weight of the clean cylinder.

10.2.8 Quantification of the number of cells

Prior to the characterization of the phenotype of the cells embedded in the biofilms, these latter were subjected to an EPS extraction procedure (sub-chapter 3.6.2). After that, the cells separated from the extracellular products were diluted to an adequate concentration, being, thereafter, stained with DAPI, according to the procedure described in sub-chapter 5.2.7.

10.2.9 Biochemical analysis

The homogenised biofilm suspensions were biochemically analyzed, being the proteins and polysaccharides determined according to the procedure described in sub-chapter 3.6.3.

10.2.10 Outer membrane proteins analysis

The analyses of the OMP were performed according to the procedure referred in sub-chapter 3.6.9.

10.2.11 Scanning electron microscopy observations

During the experiments, several stainless steel slides covered with biofilms were observed by SEM, according to the procedure described in sub-chapter 3.6.10.

10.2.12 Statistical analysis

The Student's *t* test was performed in order to validate the comparisons between the growth curves. The Wilcoxon procedure was used to compare the characteristics of both single and mixed biofilms.

10.3 Results and discussion

10.3.1 Growth profile

Figure 10.1 presents the growth curves of both *P. fluorescens* strains (D3-348, D3-350) used throughout this study.

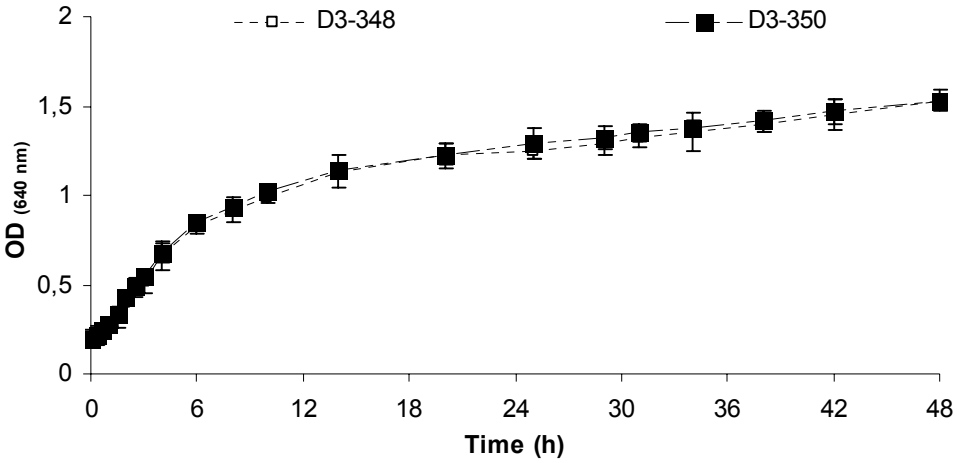


Figure 10.1 Growth curves of *P. fluorescens* D3-348 and *P. fluorescens* D3-350.

From Figure 10.1 it is possible to ascertain that both strains have a similar growth profile ($P > 0.1$), with a lag phase of about 1 h, and an exponential phase of about 6 h. Thereafter, both bacteria entered in a stationary phase. Camper *et al.* (1996), when studied the persistence of coliforms in mixed population biofilms, stated that the initial growth rate appeared to have a long-term impact on the bacterial ability to effectively compete in a mixed population biofilm. In the present study, both *P. fluorescens* strains have similar

growth profiles. So, it is expected that none of them will prevail, being coexistence the most expected behaviour when both strains are organized as mixed biofilm. However, the interactions between the populations may give rise to a biofilm with an altered behaviour.

10.3.2 Comparison of biofilms formed under different hydrodynamic conditions

The two isolated *P. fluorescens* strains were grown as single biofilms and mixed biofilms, in the flow cell reactor, operating under turbulent and laminar conditions. Figure 10.2 depicts photographs of stainless steel slides covered with biofilms formed by both strains, under turbulent and laminar flow.

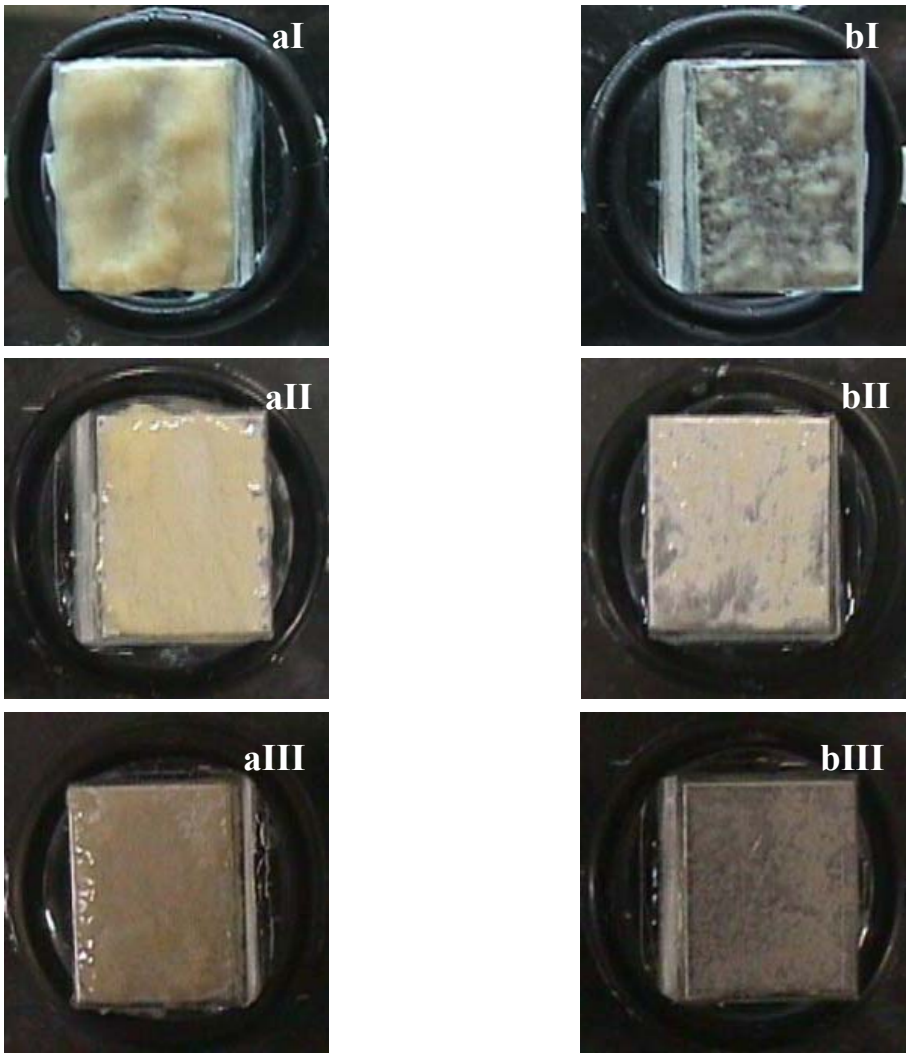


Figure 10.2 Photographs of biofilms formed on the stainless steel slides under turbulent (**a**) and laminar flow (**b**), by *P. fluorescens* D3-348 (**I**), *P. fluorescens* D3-350 (**II**) and as mixed population (**III**).

As already pointed out in Chapter 5 for biofilms formed by the type strain under the same conditions, the biofilm structure emphasized by Figure 10.2, depends on the flow conditions for both single and mixed biofilms. The turbulent biofilms appeared homogeneous and more slimy while the laminar were more heterogeneous, because biofilms were scattered on the surface, except for biofilms formed by *P. fluorescens* D3-350 where their mass covered more homogeneously the surface.

Table 10.1 presents some biofilm characteristics, namely the respiratory activity *per* mg of biofilm, the biofilm mass, the number of cells *per* mg of biofilm and *per* adhesion area, and the amount of proteins and polysaccharides present in the biofilm matrix, as well as, in the cells from the biofilms formed under different flow conditions by both *P. fluorescens* strains, as single and mixed communities.

Table 10.1 Phenotypic characteristics of biofilms grown under turbulent and laminar flow by *P. fluorescens* D3- 348 and *P. fluorescens* D3-350 as single and mixed communities. Mean ± SD

		Biofilm					
		Turbulent (D3-348)	**Turbulent (D3-350)**	**Turbulent (Mixed biofilm)**	**Laminar (D3-348)**	**Laminar (D3-350)**	**Laminar (Mixed biofilm)**
Respiratory activity ($mg\ O_2/g_{biofilm}.min$)		0.219 ± 0.043	0.273 ± 0.049	0.236 ± 0.028	0.122 ± 0.008	0.124 ± 0.013	0.0944 ± 0.021
Biofilm mass (mg/cm^2)		1.72 ± 0.33	1.16 ± 0.74	1.76 ± 0.32	1.11 ± 0.02	0.860 ± 0.16	0.940 ± 0.15
Cellular number ($cells/mg_{biofilm}$)		$6.39\times10^{10} \pm 2.8\times10^{10}$	$4.33\times10^{9} \pm 1.7\times10^{9}$	$1.33\times10^{10} \pm 6.3\times10^{9}$	$3.50\times10^{10} \pm 1.7\times10^{10}$	$2.97\times10^{9} \pm 9.1\times10^{8}$	$9.88\times10^{9} \pm 3.2\times10^{8}$
Cellular number ($Cells/cm^2$)		$1.10\times10^{11} \pm 2.9\times10^{10}$	$5.02\times10^{9} \pm 2.3\times10^{8}$	$2.34\times10^{10} \pm 8.7\times10^{8}$	$3.89\times10^{10} \pm 1.9\times10^{9}$	$2.55\times10^{9} \pm 1.8\times10^{8}$	$9.29\times10^{9} \pm 3.2\times10^{8}$
Proteins ($mg/g_{biofilm}$)	**Total**	85.6 ± 8.3	97.2 ± 7.8	101 ± 16	42.3 ± 4.5	62.8 ± 3.4	75.7 ± 11
	Matrix	21.9 ± 0.45	18.2 ± 0.30	23.2 ± 6.3	16.7 ± 0.35	16.1 ± 2.8	30.3 ± 8.3
	Cells	63.7 ± 7.2	79.0 ± 16	77.8 ± 11	25.6 ± 1.1	46.7 ± 15	45.4 ± 3.5
Polysaccharides ($mg/g_{biofilm}$)	**Total**	67.1 ± 3.2	63.5 ± 9.8	76.1 ± 13	73.5 ± 5.4	31.8 ± 1.4	61.6 ± 8.3
	Matrix	33.9 ± 0.45	33.4 ± 1.5	34.4 ± 7.7	58.2 ± 2.0	21.1 ± 7.0	41.4 ± 4.4
	Cells	33.2 ± 4.1	30.1 ± 3.3	41.7 ± 9.6	15.3 ± 1.2	10.7 ± 3.6	20.2 ± 5.2

From this phenotypic characterization, it can be said that, for both single and mixed biofilms, the turbulent biofilms were more active and had more mass *per* cm^2 than the laminar ones. Concerning the biochemical composition, turbulent biofilms had a higher content of total proteins probably due to the presence of more cells within the biofilm *per* area of adhesion, and the amount of proteins in the biofilm matrix was almost similar for both turbulent and laminar biofilms. The amount of polysaccharides was similar for both types of biofilms, except for *P. fluorescens* D3-350, where their laminar biofilms had about two times less polysaccharides *per* gram of biofilm than the ones formed under turbulent flow. Regarding the distribution of the polysaccharides in the turbulent biofilms, its concentration was similar in the matrix and in the cell composition. For laminar biofilms, the amount of polysaccharides was higher in the biofilm matrix than in the cells. Similar results were found for the *P. fluorescens* type strain studied in Chapter 5.

Comparing the characteristics of the three biofilms grown under turbulent flow, and taking into account the standard deviation values, the values of respiratory activity were very related. Concerning biofilm mass, the biofilms formed by *P. fluorescens* D3-348 and by the mixture of both strains presented similar values. The biofilm formed by *P. fluorescens* D3-350 is the one that presents the smallest amount of biofilm mass, being this phenomenon, probably, related, once more, with the lower number of cells found in the biofilm mass and *per* area of adhesion. This data may be related with the fact that *P. fluorescens* D3-348 was the microorganism that formed biofilms with the highest number of cells. The biochemical composition of the biofilms formed by the two strains as single and mixed communities, showed that all the three types of biofilms were constituted by similar amounts of proteins and polysaccharides, specially taking into account the standard deviation values. The proteins of the biofilm matrix represented about 20 % of the total proteins in the biofilm, while the polysaccharides of the matrix are about 50 % of the total amount of polysaccharides.

The characteristics related to the respiratory activity and mass were similar for the three types of biofilms grown under laminar flow, besides a slighty preponderance was detected for the biofilms formed by *P. fluorescens* D3-348. Concerning the cellular number, the same trend found for turbulent biofilms happened for the laminar ones. The analysis of the biochemical composition shows variation between the single biofilms and the mixed biofilm. Mixed biofilms had the highest amount of total proteins as well as the proteins of the matrix. However, their relative proportions of the proteins of the matrix and the proteins

of the cells, respectively, 40 % and 60 %, are similar to the ones found for the biofilms formed by *P. fluorescens* D3-348. Nevertheless, this latter biofilm presented the smallest amount of total proteins. Concerning polysaccharides content, the biofilms formed by *P. fluorescens* D3-348 presented the higher total polysaccharides content, in which 80 % were in the matrix. The relative percentage of the polysaccharides of each biofilm fraction (matrix and cells) of the biofilms formed by *P. fluorescens* D3-350 was very close to the mixed biofilms, but different from the ones determined for the biofilms formed by *P. fluorescens* D3-348.

The biochemical differences observed within the biofilms (single and mixed) could be a phenomenon related with the number of cells and with the cellular state, as pointed out for the type strain in Chapter 5. Concerning the mixed biofilms, it was not found any clear characteristic as a consequence of the interactions promoted by the two strains during biofilm formation that evidenced significant differentiation in this biofilm. The biofilm presents characteristics that seem to be an intermediate of the values presented by each single biofilm.

Figure 10.3 displays SEM microphotographs representative of the several fields observed in each biofilm-covered metal surface.

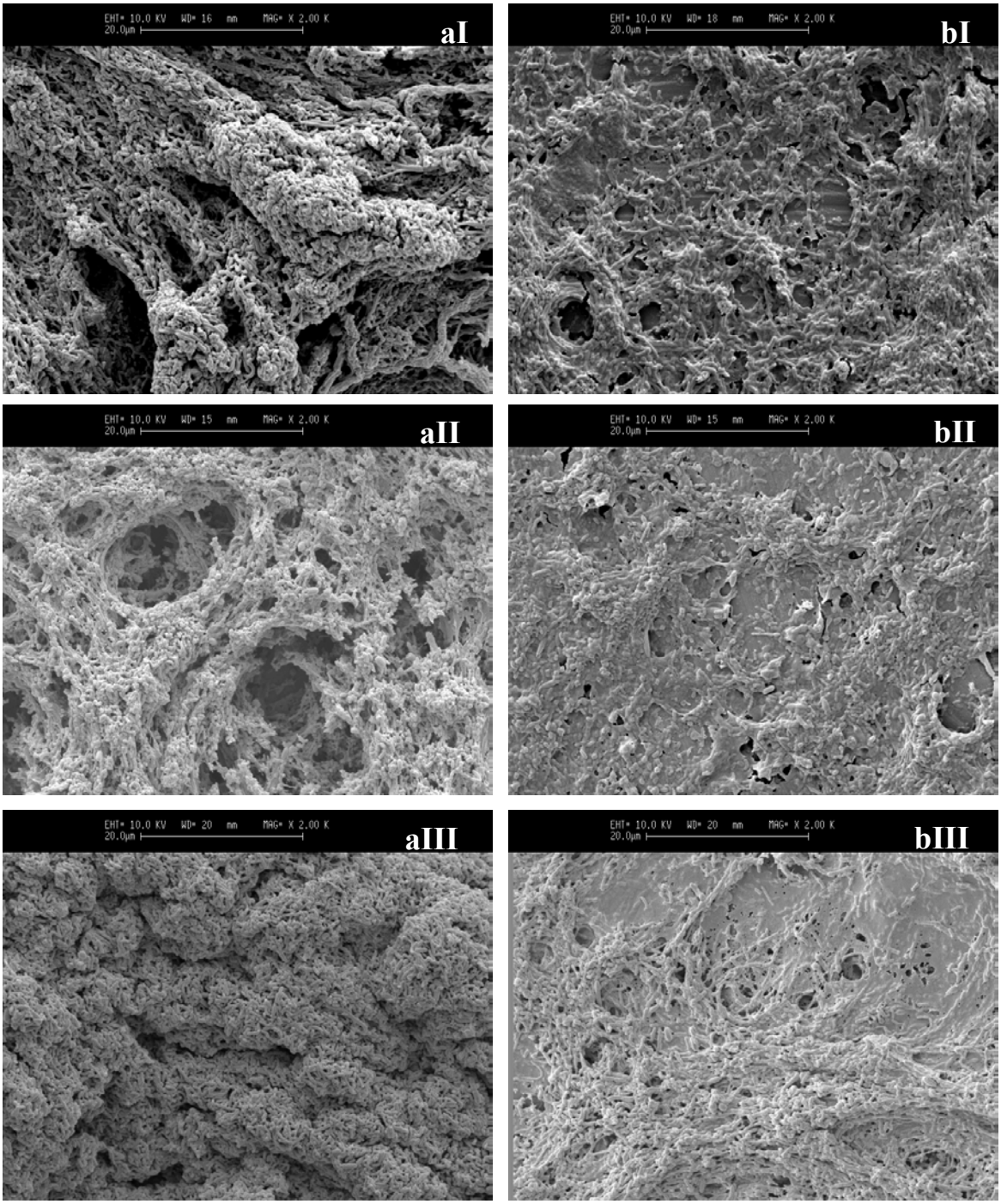


Figure 10.3 Scanning electron microscopy photomicrographs of a 7 d old biofilms formed on stainless steel slides under turbulent (**a**) and laminar (**b**) flow by *P. fluorescens* D3-348 (**I**), *P. fluorescens* D3-350 (**II**) and by both strains (**III**). X 2000 magnification, bar = 20 μm.

As previously verified in Chapter 5 for the type strain, the SEM analysis revealed that the biofilm structure depends on the flow conditions. Additionally, some differences are encountered when comparing the different strains and biofilm type (single or mixed) for the same hydrodynamic condition. These differences can be, in part, associated with the

different biofilm characteristics emphasized by Table 10.1. For instance, the structure of the biofilms formed under turbulent flow by *P. fluorescens* D3-350 (Figures 10.3 aII), reveals the existence of water channels that may have favoured the diffusion of nutrients. This biofilm characteristic could account for the more active metabolic state of the cells within the biofilm. In fact, biofilms formed by *P. fluorescens* D3-350 under turbulent flow were the ones that present the highest respiratory activity (Table 10.1) even though cells were in a smaller extent.

10.3.3 Outer membrane proteins

The OMP of the two *P. fluorescens* strains, as planktonic cells and sessile cells (the latter developed within turbulent and laminar biofilms) were isolated and analysed by SDS-PAGE. These OMP profiles obtained are presented in Figure 10.4.

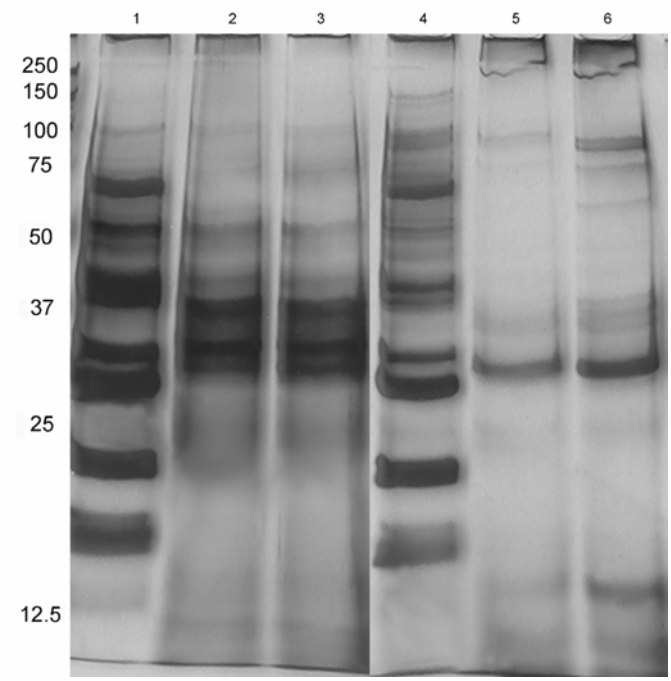


Figure 10.4 Outer membrane proteins profile of planktonic cells and cells within biofilms formed under turbulent and laminar flow regimes: OMP from *P. fluorescens* D3-348 cells in planktonic state (lane 1) and within biofilms formed and turbulent (lane 2) and laminar (lane 3) flow; OMP from *P. fluorescens* D3-350 cells in planktonic state (lane 4) and within biofilms formed and turbulent (lane 5) and laminar (lane 6) flow. Numbers on the left represent molecular weights in kDa.

The OMP profiles obtained with cells developed within biofilms differ considerably from the ones obtained with their free cell counterparts for both strains studied. The OMP profiles of both strains in the planktonic state are; however, similar. Cells within biofilms expressed a lesser number of OMP than the ones in planktonic state, specially, those proteins with approximately 18 and 23 kDa. The different flow regimes implemented for biofilm formation seems no to cause OMP change since, for both strains tested, the protein expression is comparable. This result is in accordance with the one obtained with the type strain (Chapter 5). The comparison of the OMP profiles of the biofilm cells, for the two strains, reveals that the OMP expression is relatively similar.

The changes in the OMP expression and in the general phenotype of cells within biofilms when compared with their freely counterparts is a phenomenon already observed in Chapter 5, and documented by other authors (Coquet *et al.,* 2002; Sauer *et al.,* 2002). The flow regime under which the biofilms were formed seems not to be accountable for the OMP changes, since cells within the different biofilms present similar OMP profiles.

10.3.4 Biofilm formation on the bioreactor rotating system

A phenotypic study of the cells from the single biofilms and from the mixed biofilms formed after 7 d of growth in the rotating device at a $N`Re_A$ of 2420 was carried out.

Figure 10.5 shows stainless steel cylinders covered with biofilms formed by the two strains as single and mixed biofilms.

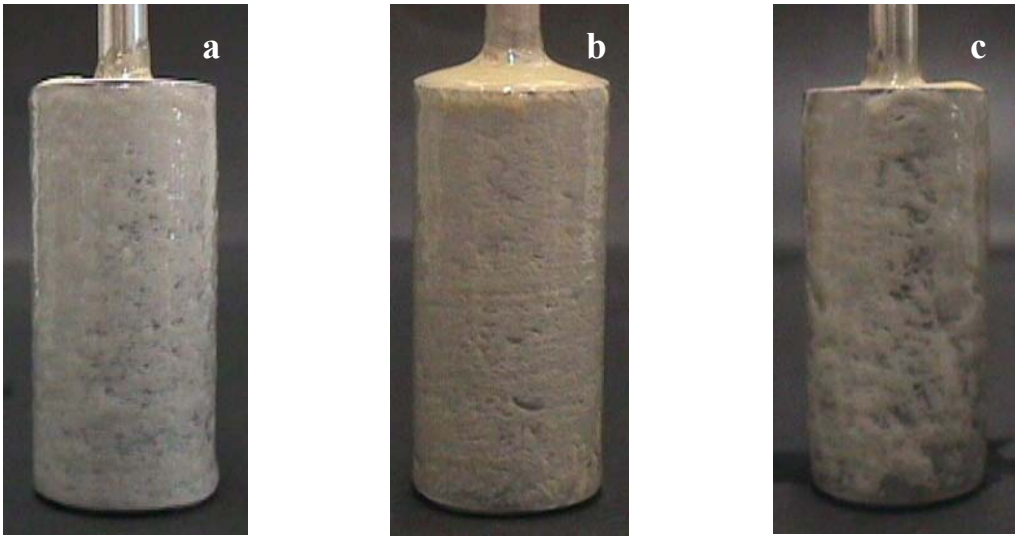


Figure 10.5 Stainless steel cylinder covered with biofilm after 7 d of growth formed by *P. fluorescens* D3-348 (**a**), *P. fluorescens* D3-350 (**b**) and by simultaneously both strains (**c**).

Figure 10.5 clearly shows that the surface area of the stainless steels cylinders were completely covered with biofilm, regardless the strain or biofilm type. As well as for the type strain (Chapter 9), it is noticeable the existence of a thick and slimy biofilm that seems to be strongly adhered to the surface. Comparing the three biofilms presented in this Figure, it seems that they present some differences in their macroscopic surface. It is clear that the surface of the mixed biofilm is different from the single biofilms.

As already carried out for the type strain, some characteristics of the biofilms formed on the cylinders of the rotating device, namely the biofilm activity, mass, proteins and polysaccharides content, are presented in Table 10.2.

Table 10.2 Phenotypic characteristics of the biofilms formed on the surface of the stainless steel cylinders as single and mixed biofilms

		Biofilms		
		D3-348	**D3-350**	**Mixed biofilm**
Biofilm activity ($mg\ O_2/g_{biofilm}.min$)		0.330 ± 0.04	0.165 ± 0.06	0.253 ± 0.09
Biofilm mass (mg/cm^2)	**Dry**	0.485 ± 0.10	0.516 ± 0.14	0.501 ± 0.04
	Wet	21.0 ± 1.1	19.3 ± 3.3	21.5 ± 2.9
Proteins ($mg/g_{biofilm}$)	**Total**	288 ± 15	188 ± 4.3	215 ± 33
	Matrix	61.3 ± 7.8	49.8 ± 84	75.2 ± 4.4
	Cells	227 ± 19	138 ± 11	140 ± 14
Polysaccharides ($mg/g_{biofilm}$)	**Total**	219 ± 11	201 ± 22	231 ± 15
	Matrix	130 ± 24	108 ± 31	153 ± 20
	Cells	89.3 ± 9.4	93.4 ± 12	78.0 ± 5.7

From Table 10.2 it can be noticed that the three biofilms were metabolically active and contained about 98% of water. The biofilms formed by *P. fluorescens* D3-348 were the ones that, even though presented higher values of respiratory activity, had less mass and total amount of proteins. Conversely, a different trend was found for the biofilms formed by *P. fluorescens* D3-350. For the mixed biofilm, the values of respiratory activity, mass and total amount of proteins seem to be an intermediate of the characteristics of the two single biofilms, as already pointed out for the biofilms formed in the flow cell reactors. Concerning the amount of polysaccharides, it is quite similar for the three biofilms. Besides the differences found for the total amount of proteins and polysaccharides, the proportion of

proteins and polysaccharides in the matrix is almost similar for single biofilms, respectively about 25 % for proteins and about 55 % for the polysaccharides, but different for the mixed biofilms (about 35 % of the total protein and 66 % of the total polysaccharides).

Comparing the characteristics of the biofilms presented in this chapter with the ones formed by the type strain (Chapter 5), several differences are encountered. In terms of respiratory activity, only the biofilms formed by *P. fluorescens* D3-350 presented similar activity to the ones formed by the type strain, while the other biofilms were about two times more active. The biofilm mass is quite similar for the three biofilms studied in this chapter, being their biofilm mass half of the one assessed for the type strain. Concerning the biochemical composition, the type strain had similar amount of total, extracellular and cellular proteins and polysaccharides when compared with the biofilms formed by *P. fluorescens* D3-350 and the mixed biofilm. *P. fluorescens* D3-348 formed biofilms with a higher content of the proteins, being 80 % proteins of cells, which can be related, probably, with the existence of a higher number of cells, as previously found for the biofilms formed in the flow cell reactor by this strain. However, a significant content of extracellular proteins and polysaccharides was found in the composition of the biofilm matrix. This feature of the biofilm has a strong importance in the establishment of the biofilm mechanical stability (Azeredo and Oliveira, 2000; Körstgens *et al.,* 2001).

10.3.5 Comparison of the behaviour of the biofilms when exposed to external mechanical stress conditions

The mechanical stability of biofilms, previously defined in Chapter 9 as the behaviour of biofilms face to external stress mechanical conditions, was characterized.

Figure 10.6 shows the biofilm removal obtained due to the increase in the rotation speed of the cylinder (expressed as $N`Re_A$) for each biofilm studied.

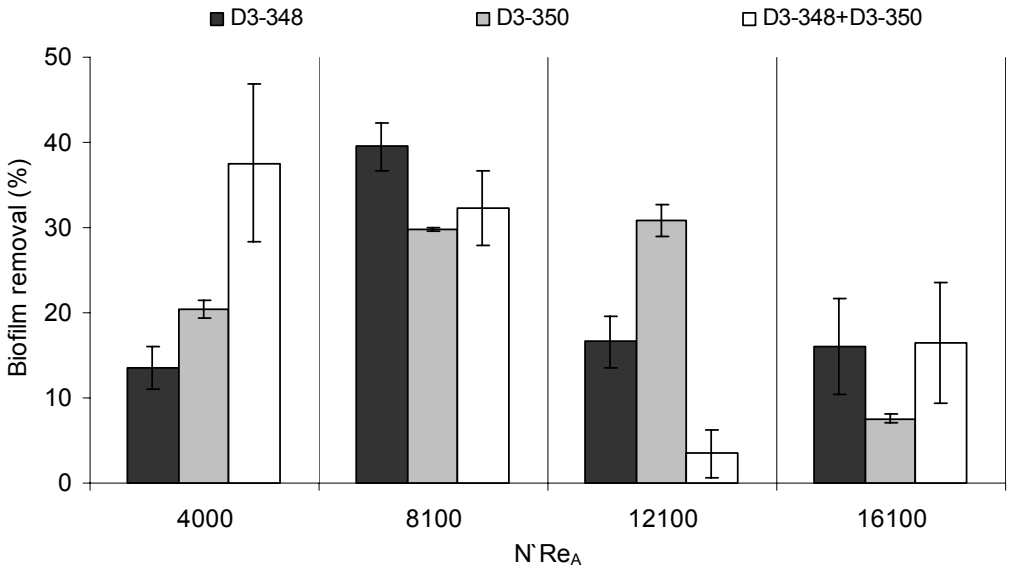


Figure 10.6 Biofilm removal of the different biofilms studied due to the exposure to increasing $N`Re_A$.

As previously observed for the type strain (Chapter 9), the exposure of the biofilms to $N`Re_A$ higher than the one under which the biofilm was formed (*i.e.* 2420) caused biofilm removal. The biofilm matrix develops an inherent internal tension, which is in equilibrium with the shear stress under which the biofilm was formed (Stoodley *et al.* 1999), and the removal of a well established biofilm requires the overcome of the forces that keep the biofilm matrix together as well as the forces that bound the biofilm to the adhesion surface (Körstgens *et al.* 2001). These statements are in accordance with this study since, for the different biofilms, the higher percentage of removal happened with the exposure of biofilms to $N`Re_A$ of 8100, 12100 and 4000 respectively for biofilms formed by *P. fluorescens* D3-348, *P. fluorescens* D3-350 and for the mixed biofilms. The statistical treatment shows that only for the mixed population the biofilm removal was dependent on the $N`Re_A$ ($P < 0.05$). Comparing with the results obtained for the type strain, the three biofilms had similar removal behaviour ($P > 0.1$) in terms of biofilm removal. The submission to the increasing $N`Re_A$ did not give rise to total biofilm removal, since only about 76 % (*P. fluorescens* D3-348), 88 % (*P. fluorescens* D3-350) and 90 % (mixed) of biofilm mass were detached from the cylinders, indicating that the mechanical treatment was not enough to eradicate the biofilms (Table 10.3), as assessed for the type strain (Chapter 5).

Table 10.3 Biofilm remaining for the single and mixed biofilms posterior to the exposure to the total series of N`Re_A

Biofilm remaining (%)		
D3-348	**D3-350**	**Mixed biofilm**
24.1 ± 1.7	11.5 ± 3.2	10.1 ± 3.4

Table 10.3 emphasize that it is not possible to remove all the biofilm by sole mechanical treatment, being this a proof of the inherent mechanical stability of the biofilms, corroborating the results presented for the type strain. Nevertheless, it was found heterogeneity in the values of biofilm remaining within the biofilms. The biofilms formed by *P. fluorescens* D3-348 presented a higher mechanical stability than the ones formed by *P. fluorescens* D3-350 and the mixed biofilm, being the percentage of biofilm remaining equal to the one left on the surface by the biofilms formed by the type strain (24 %). Concerning the comparison of the values of biofilm remaining for the biofilms formed by *P. fluores*cens D3-350 and the mixed biofilms the similarity could be due to the prevalence of this strain in the inner layers of the mixed biofilm, since biofilm detachment is processed in layers (Azeredo and Oliveira, 2000). However, interactions between both strains could give rise to biofilms with a diminished mechanical strength, because about 90 % of the mixed biofilms were removed by using the smaller N`Re_A (4000). However, the remaining 10 % of the initial biofilm could promote the regrowth of a biofilm with more recalcitrant properties than the one that was initially exposed to the mechanical treatment.

10.4 Conclusions

The choice of the correct bacterial strain that represents the actual industrial environment, where the biofilms are a problem, should be a concern when the laboratorial experiments are designed.

The hydrodynamic conditions under which biofilms were formed and the sessile mode of growth had a significant impact in the biofilm metabolic activity, mass formation ability, biochemical composition and OMP expression (when compared with the planktonic situation).

The results presented in this chapter allow to conclude that the characteristics of a strain from a bacterial specie and the presence of different strains in a biofilm may account

for differences in biofilm structure and behaviour. However, such differences are attenuated when comparing biofilms formed by different strains and communities (single/mixed).

The studies made with the *P. fluorescens* type strain are applicable to strains encountered in industrial systems. In fact, similar characteristics were found in biofilms from the different strains, regardless the biofilm community (single/mixed). Another feature that reinforces this idea is that the mechanical stability of the biofilms formed by the type strain presented the higher recalcitrance to removal face to the mechanical stress.

Chapter 11

Concluding remarks and perspectives for further research

Abstract

This chapter presents the general conclusions of this thesis and identifies future research to advance the optimization of methods to control unwanted biofilms.

11.1 General conclusions

From the work presented in this thesis some major conclusions can be withdrawn:

The use of different methods to evaluate the efficacy of antimicrobial agents can lead to different conclusions regarding the effects caused by the chemicals. However, it was concluded that respiratory activity using oxygen consumption rate represents an expeditious, non-destructive and accurate method to determine the antimicrobial action of biocides against aerobic heterotrophic bacteria.

P. fluorescens respond to changes in their immediate environment (planktonic/ biofilm state and hydrodynamic conditions) by a remarkable phenotypic plasticity involving changes in their physiology, their cell composition and structure and their resistance to antimicrobial agents. This level of structural organization and metabolic specialization help to explain the remarkable metabolic efficiency of cells within biofilms and their resistance to antimicrobial agents. The flow conditions under which the biofilms were formed had a significant impact in the biofilms characteristics and behaviour and thus in the consequent response to the action of antimicrobial agents. Biofilms formed under laminar flow were more easily inactivated than the ones formed under turbulent conditions. OPA was the only antimicrobial tested that promoted total inactivation of biofilms (laminar biofilms). With the experimental systems used, detachment was found to be a significant slower process than biofilm inactivation.

A failure to effectively disinfect, with the aldehyde-based biocides and with the surfactants, leaded to biofilm population experienced at responding to toxic stress, which facilitated the development of resistant population with capability to recover their metabolic state.

Studies with planktonic cells provide an incomplete picture of antimicrobial action when used as model to be applied against a biofilm. The behaviour of bacterial cells face to a toxic environment was significantly different when the cells were in suspension or when they were embedded in a biofilm. This study corroborates the nearly universally observed resistance of biofilm microorganisms to disinfection when compared with their freely suspended counterparts. Conversely to the European Standard (EN 1276) that proposes to

assay cleaning and disinfection agents using cells in suspension, this thesis clarifies that bacteria within a biofilm are much more resistant to antimicrobial agents than planktonic cells. On the other hand the antimicrobial potential of the chemicals was drastically quenched by the existence of dirty conditions.

The effect of the chemical compounds on the biofilm removal and mechanical stability varied with the chemical nature of the antimicrobial agent, being chemical treatment far from a factor that induced massive detachment. Even the synergistic chemical and mechanical treatment did not induced total biofilm eradication, proposing that care must be taken and experiments must be made concerning the choice of the correct protocol for biofilm control. GTA and SDS at the lower concentrations induced the increase in the biofilm mechanical stability. OPA did not have a significant effect on the biofilm removal and consequent mechanical stability. CTAB, NaOH, SHC and SDS at concentrations near the CMC induced higher removal due to mechanical stress and thus decreased the biofilm mechanical stability.

The choice of a correct strain, for laboratory experiments, representative of a phenomenon existent in a particular process or representative of specie, is a fundamental step in the development of reliable procedures for biofilm control. The type strain of *P. fluorescens* demonstrated to be a broad spectrum microorganism of the specie when compared with two strains isolated from a dairy process. Thus it can be a good starting point when it concerns studies of *P. fluorescens* biofilm formation and control.

The experimental systems used (flow cell and rotating bioreactor) were suitable for the evaluation of biofilm growth, and represented very useful tools to perform biofilm studies.

11.2 Perspectives for further research

Much more needs to be learned about the impact of antimicrobial agents on bacteria and their response to the damage.

The significance of biofilms is not a well understood phenomenon because of a lack of direct observation of biofilms in their environment and a lack of research using model systems that closely simulate the environmental system. Most investigations involving biofilms have been performed using *in vitro* laboratory models. However, all these *in vitro* models of biofilms meet serious difficulties to sort out the contribution of important individual parameters. One of the keys to study complex biological systems seems to be the development of accurate and realistic models of natural communities in the laboratory. With this purpose the identification of bacteria forming natural biofilms seems to have utmost magnitude. Subsequently, the assessment of the potential of the bacteria for biofilm formation as single and mixed species biofilms and the evaluation of the interspecies interactions, probably, can provide new data in order to understand the phenomenon behind biofilm recalcitrance and will provide new mechanisms for biofilm self-regulation. So, the intense dissection of these interactions provides one of the future challenges in biofilm research/control.

The environment changes appeared to influence the biofilm structure and activity, where this complex biofilm architecture obviously provides an opportunity for metabolic cooperation and niches are formed within this spatial well-organized system. Consequently, an understanding of the structure-function relationships in microbial biofilms seems to be fundamental to interpret and predict biofilm impacts on the habitat where they are developed.

Since the altered phenotype is believed to be responsible for the distinct properties of bacteria growing in biofilms and it has been also suggested that it is related with the enhanced resistance of biofilm cells to antimicrobial agents, so it will be decisive to find about the adaptative mechanisms involved in the bacterial resistance to antimicrobial compounds as well as other forms of stress. So, it will be required to identify the essential proteins involved in the enhanced resistance of cells within biofilms to antimicrobial agents, and to research suitable modes of their repression. The analysis of the microbial resistance

to antimicrobial agents will be assessed using proteomic techniques, by the identification of target proteins that are necessary to the biofilm state, *i.e.,* proteins that are common to the biofilm state among different adherent species and whose synthesis inhibition will prevent biofilm formation or accelerate biofilm eradication.

The use of certain antimicrobial agents can impose a selective pressure and can contribute to the emerge of resistant microorganisms. Therefore, these microorganisms can become resistant to other types of antimicrobial agents, causing serious problems to the process under study. So, investigation about the cross-resistance between antimicrobial agents may become a challenge.

Nowadays, the modern view of biofilm formation has lead to the realization that effective control of biofilms will require the development of agents that are community signalling-based and/or that target the biofilm phenotype (ex. target biosynthesis of the EPS matrix and/or signal molecules associated with the sensing of surfaces proximity or high localized cell densities). However, it will be required that such agents follow the specifications needed to be used in a process (ex. dairy industry).

The realization of this future work perspective is enclosed in a project (CHEMBIO II – Chemical and Biological Control of Biofilms II) and in my Pos-Doc program, both already approved by the Portuguese Foundation for Science and Technology.

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