FUNCTIONALIZED MICROPARTICLES FOR ONLINE BIOFILM CONTROL

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"We ourselves feel that what we are doing is just a drop in the ocean. But the ocean would be less because of that missing drop."

Madre Teresa de Calcutá

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Ferreira C., Simões M., Pereira M. C., and Melo L. F., Calcium carbonate microparticles as an antibiofouling biocide carrier, Heat Exchanger Fouling and Cleaning 2013, Budapest.

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Ferreira C., Simões M., Pereira M. C., Bastos M.M.S.M., Nunes O.C., Coelho M. and Melo L. F., Control of biofouling of industrial surfaces using microparticles carrying a biocide, Heat Exchanger Fouling and Cleaning 2009, Austria, 6pp, 2009.

Poster communications in international conferences:

Ferreira C., Rosmaninho R., Pereira M. C., Bastos M.M.S.M., Nunes O.C., Coelho M. and Melo L. F., Functionalized nano-microstructures to combat biofouling of industrial surfaces, Nanospain 2008 – NanoIberian Conference, Braga April 2008.

Ferreira C, Simões M., Pereira M.C., Bastos M.M.S.M., Nunes O., Coelho M. and Melo L.F. Biofilm treatment with functionalized antimicrobial microparticles, The Water Research Conference, Lisbon, April 2010.

Abstract

Biofilms are ubiquitous in nature and its formation is a strategy that bacteria use in order to survive in hostile environments. Although biofilms play crucial roles in many processes they often are unwanted and cause serious problems in various areas like the food industry, medical industry, industrial heat exchangers and cooling water systems, which can lead to increase in the costs of production and maintenance, as well as to public health and environmental concerns and impacts.

Although various methods of biofilm control exist, these present limitations and often fail to remove completely the biofilms from surfaces, contributing to the dissemination of resistance. The control of undesirable biofilms often include the use of chemical products with antimicrobial properties such as biocides and surfactants. However, these substances can be harmful to the environment and public health, therefore they should be used in as small quantities as possible. In the past few decades there has been a dilemma between effective disinfection and formation of harmful disinfection by-products (DBPs).

Advances in nanotechnology science led to its environmental application. The focus of this work was the development of microparticles with functionalized surfaces that carry the antimicrobial agent. This strategy significantly reduces the amount of antimicrobial agent needed, prevents undesired reactions with other components that can lead sometimes to the formation of organochlorinated compounds (potentially carcinogenic), and finally reduces the costs of wastewater treatment due to the presence of residual concentrations of biocides in the effluent.

In the beginning of this study, the action of the quaternary ammonium compound benzyldimethyldodecylammonium chloride (BDMDAC) was assessed against the Gram-negative bacterium *Pseudomonas fluorescens*. The targets of antimicrobial action were studied using different bacterial physiological indices. The minimum inhibitory concentration (MIC) was 20 mg/L and the minimum bactericidal concentration (MBC) value was 10 mg/L. BDMDAC led to a significant change in cell surface hydrophobicity and induced propidium iodide uptake. Such results suggest cytoplasmic membrane damage, corroborated by the release of intracellular potassium. The results obtained from the zeta potential assay indicated the existence of interactions between the cationic antimicrobial agent and the cell surface. Scanning electron microscopy (SEM) allowed

to detect that cells treated with 20 mg/L were less bulky and their membrane seems to be rougher, wrinkled, deformed comparatively to untreated cells.

The microparticles of polystyrene (PS) were functionalized with BDMDAC using the layer-by-layer (LbL) self-assembly technique. Zeta potential measurements indicated significant changes in the surface charge compatible with the BDMDAC/particle interaction. The efficacy of microparticles carrying BDMDAC was assessed against *P. fluorescens* in planktonic and biofilm states. This biocidal carrier structure had a significant stability verified by the release of only 15% of BDMDAC when immersed in water for 18 months. The evaluation of biocidal carrier activity was carried out through the determination of the survival ratio of *P. fluorescens* planktonic and biofilm cells after different exposure periods to BDMDAC coated particles. Tests with biofilm cells were also performed with free BDMDAC. MBC of BDMDAC coated polystyrene (PS) microparticles was 9.2 mg/L and 6.5 mg/L for exposure periods of 30 and 60 minutes, respectively. Biofilms were developed in a chemostat reactor and polyvinylchloride was used as adhesion surface. Seven days old biofilms were exposed to BDMDAC coated PS microparticles (0.87 mg/L) resulting in viability decreases of 60.5% and 66.5% of the total biofilm population for a 30 and 60 min exposure time, respectively. The exposure during 60 min to 6.33 mg/L and 11.75 mg/L of BDMDAC in PS coated microparticles promoted inactivation of 80.6% and 87.2% of the total population, respectively. The antimicrobial effects obtained with the application of free BDMDAC were similar to those promoted by the application of BDMDAC coated particles.

Due to the high cost of PS microparticles, calcium carbonate ($CaCO_3$) microparticles were selected for testing BDMDAC immobilization with the LbL assembly technique. These microparticles have the advantage of being cheaper and porous (and the disadvantage of being susceptible to low pH values). *P. fluorescens* biofilms were exposed to 6.33 mg/L and 11.75 mg/L of BDMDAC-coated $CaCO_3$ microparticles for 60 min. This strategy promoted inactivation of 81.9% and 93.3% of the total population.

The effects of flowing conditions on biofilm susceptibility to BDMDAC-coated $CaCO_3$ microparticles was tested using a flow cell reactor, mimicking industrial pipes, for biofilm formation and antimicrobial testing. SEM images of the microparticles show that they are spherical and have a spongy surface. These microparticles in suspension

have an average size diameter of 5 μm and a zeta potential of -20 ± 3 mV. The exposure of biofilms to 50 mg/L of BDMDAC-coated $CaCO_3$ microparticles promoted inactivation of 100% of the biofilm population after 90 minutes of exposure. The overall results indicate that this novel strategy has a strong potential to control flow-generated biofilms and has many advantages over traditional biofilm control strategies, particularly the possibility for the reuse of the biocidal particles and consequent reduction of discharges of antimicrobials into the environment.

Resumo

Os biofilmes são omnipresentes na natureza e a sua formação é uma estratégia que as bactérias usam para sobreviver em ambientes hostis. Embora desempenhem um papel crucial em muitos processos, a presença de biofilmes é muitas vezes indesejada. Quando associados às indústrias alimentar e médica, aos sistemas de refrigeração de água e permutadores de calor industriais, a sua presença traduz-se no aumento dos custos de produção e manutenção, bem como no desenvolvimento de potenciais riscos para a saúde pública e para o ambiente.

Embora existam vários métodos de controlo dos biofilmes, estes apresentam algumas limitações e, na maioria das vezes, não conseguem remover completamente os biofilmes, contribuindo para a disseminação da resistência microbiana. O controlo de biofilmes indesejáveis inclui o uso de produtos químicos com propriedades antimicrobianas, tais como agentes tensioativos e biocidas. No entanto, estas substâncias podem ser prejudiciais para o ambiente e para a saúde pública, limitando a sua aplicação em grandes quantidades. Nas últimas décadas tem havido controvérsia no que diz respeito à desinfeção eficaz e à formação de subprodutos nocivos resultantes das desinfeções.

Atualmente os avanços na área da nanotecnologia possibilitaram a sua aplicação na área ambiental. Desta forma, o objetivo deste trabalho foi o desenvolvimento de micropartículas com superfícies funcionalizadas que transportam o agente antimicrobiano ou biocida. Esta estratégia reduz significativamente a quantidade de agente antimicrobiano necessária, impede reações indesejadas com outros componentes, que podem levar à formação de compostos organoclorados (potencialmente carcinogénicos), e, por último, reduz os custos associados ao tratamento de águas, devido à presença de concentrações residuais de biocida nos efluentes.

Inicialmente foi avaliado o mecanismo de ação do biocida cloreto de benzildimetildodecilamónio (BDMDAC) em relação à bactéria Gram-negativa *Pseudomonas fluorescens*. A ação antimicrobiana foi estudada utilizando diferentes índices fisiológicos bacterianos. A concentração mínima inibitória (MIC) foi de 20 mg/L e o valor da concentração mínima bactericida (MBC) foi de 10 mg/L. A utilização deste biocida levou a uma alteração significativa da hidrofobicidade de superfície celular e, para concentrações crescentes de BDMDAC, observou-se um aumento na absorção de

iodeto de propídio. Tais resultados sugerem danos na membrana citoplasmática, que foram comprovados pela liberação de potássio intracelular. A partir do ensaio de potencial zeta verificou-se a existência de interações entre o agente antimicrobiano catiónico e a superfície da célula. A microscopia eletrónica de varrimento (SEM) permitiu visualizar que as células tratadas com 20 mg/L de BDMDAC apresentam um volume menor e que a sua membrana aparenta ser mais áspera, enrugada e deformada que a das células não tratadas.

Posteriormente, foram funcionalizadas micropartículas de poliestireno (PS) com BDMDAC usando a técnica de camada sobre camada. Através das medições de potencial zeta foi possível verificar alterações significativas na carga da superfície compatível com a interação partícula/BDMDAC. A eficácia das micropartículas funcionalizadas com BDMDAC foi testada em *P. fluorescens* em estado planctónico e em biofilme. A estabilidade das micropartículas utilizadas para suporte e transporte do biocida foi comprovada porque ao fim de 18 meses imersas em água, apenas 15% de BDMDAC foi libertado. Após diferentes períodos de exposição às micropartículas funcionalizadas, determinou-se a razão de sobrevivência da bactéria *P. fluorescens*, tendo-se realizado um controlo com biocida livre. Para períodos de exposição de 30 e 60 minutos, a MBC das micropartículas de PS revestidas com BDMDAC foi de 9,2 mg/L e 6,5 mg/L, respetivamente. Os biofilmes foram desenvolvidos num quimiostato usando cupões de cloreto de polivinilo como superfície de adesão. Após 7 dias de formação de biofilme, estes foram expostos a micropartículas funcionalizadas com BDMDAC (0,87 mg/L), o que resultou numa diminuição de viabilidade de 60,5% e 66,5% para tempos de exposição de 30 e 60 min, respetivamente. A exposição durante 60 minutos a 6,33 mg/L e 11,75 mg/L de biocida imobilizado promoveu uma inativação de 80,6% e 87,2% da população total, respetivamente. O efeito antimicrobiano obtido com a aplicação de BDMDAC livre foi semelhante ao efeito resultante da aplicação de micropartículas revestidas com o mesmo biocida.

Devido ao elevado custo das micropartículas de PS, foi estudada uma solução mais económica, recorrendo a micropartículas de carbonato de cálcio ($CaCO_3$) funcionalizadas com BDMDAC através da técnica camada sobre camada. Estas micropartículas têm a vantagem de serem mais baratas e porosas (e a desvantagem de serem suscetíveis a baixos valores de pH). Biofilmes de *P. fluorescens* foram expostos durante 60 min a 6,33 mg/L e 11,75 mg/L de BDMDAC imobilizado nas

micropartículas. Esta estratégia promoveu, uma inativação da população total de 81,9% e 93,3%, respetivamente.

A fim de mimetizar as tubagens industriais e respetivos biofilmes formados nesses ambientes, utilizou-se um sistema designado por célula de fluxo para o estudo da formação de biofilmes em fluxo, assim como do comportamento das micropartículas funcionalizadas nestas mesmas condições. As imagens de SEM das micropartículas mostraram que estas são esféricas e têm uma superfície esponjosa. Em suspensão estas micropartículas apresentam um diâmetro médio de 5 μm e um potencial zeta de -20 ± 3 mV. A exposição durante 90 minutos de biofilmes de *P. fluorescens* a micropartículas de $CaCO_3$ revestidas com 50 mg/L de BDMDAC promoveu uma inativação de 100%. Os resultados globais indicam que esta nova estratégia apresenta um elevado potencial no controlo de biofilmes, tendo várias vantagens sobre as estratégias tradicionais, em especial a possibilidade de reutilização das partículas biocidas e consequente redução da libertação de agentes antimicrobianos para o meio ambiente.

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List of Abbreviations

BAC	benzalkonium chloride
BDMDAC	benzlydimethyldodecylammonium chloride
CFU	colony formation units
CryoSEM	cryo scanning electron microscopy
HPLC	high performance liquid chromatography
LbL	layer-by-layer
LNEG	national laboratory of energy and geology
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
PAH	poly(allylamine hydrochloride)
PDDA	poly(diallyldimethylammonium chloride)
PEI	poly(ethyleneimine)
PEMs	self-assembled polyelectrolyte multilayers
PSS	poly(sodium styrenesulfonate)
QAC	quaternarium ammonium compounds
SEM	scanning electron microscopy
VBNC	viable but nonculturable

Chapter 1

Motivation and presentation

1.1. Motivation

Layers of microorganisms and their extracellular polymers ("biofilms") grow very easily on industrial cooling water tubes and heat exchanger channels, causing increased pressure drop and reduced heat transfer efficiency. These problems lead, ultimately, to an increase in the costs of the production and maintenance, as well as to public health problems and environmental impacts (Pereira et al., 2008). Often the layers build up in a non-uniform manner, with localized spots where thicker biofilms appear. Biofilm growth on surfaces is prevented by using biocides (e.g., chlorine, quaternary ammonium compounds, caustic agents, etc) and detergents in the water stream in considerable large amounts. According to the Directive 98/8/EC, biocides are "chemicals with an active and in general toxic effect on living organisms, 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" (Rasmussen et al., 1999). Such toxic chemicals are not totally consumed in the disinfection process and their discharge into the environment or wastewater treatment plants is a source of serious problems.

Nanotechnology permits to create artificial systems with enormous potential for numerous applications. The purpose of the project is to develop micro- and nanoparticles with functionalized surfaces that carry the biocide. This will save significant amounts of biocide, prevent undesired reactions with other components that can lead sometimes to the formation of undesirable disinfection by-products such as organochlorinated compounds (potentially carcinogenic), and finally will reduce the costs of wastewater treatment due to the presence of residual concentrations of biocides in the effluent. The mitigation of biofilm build up on industrial surfaces will also reduce pressure drop and thermal resistance in pipes and heat exchangers.

Therefore, beneficial impacts are expected with this project in terms of:

- Reduction of environmental costs due to biocide release;
- Better control of the health risks associated with the use of biocides (toxic compounds);
- Savings in energy consumption due to efficient biofouling control (reduced pumping costs and higher heat transfer efficiency);

Reduction in the costs of biocide acquisition. Furthermore, it will contribute with advancements on the fields of surface science, biofilm science and engineering, allowing: i) a better control of the micro- and nanoparticles stability in aqueous solutions; ii) the optimization of surface interactions in biological systems (particle-biofilms); and iii) the study of the effects of an immobilized biocide in biofilm control.

The foreseen advantages of this approach are that such microstructures can be functionalized to be adsorbed at the surface layer of biofilms and to penetrate deeply enough to release the biocide agent in the inner layers. This methodology has not yet been developed for industrial systems and can be used either as a cleaning or preventive technique.

1.2. Thesis organization

This thesis is outlined in 7 chapters. This chapter, *Motivation and Presentation*, covers the scope and the objectives of the proposed work. Chapter 2 presents a brief literature review about biofilm formation, strategies for biofilm control, biocides, application of micro- and nanotechnology in biofilm control. Chapter 3 presents the study of the mechanism of action of the biocide benzyldimethyldodecylammonium chloride (BDMDAC) against a strain of *Pseudomonas fluorescens*. Chapter 4 presents a development of the PS polystreyne microparticles developed and their action against the biofilm formed in a chemostat-like reactor. Chapter 5 presents a comparison of the PS microparticles and a new functionalized calcium carbonate ($CaCO_3$) microparticles coated with biocide, and assess their efficacy against biofilm formed in a chemostat reactor. Chapters 6 presents the study of the efficacy if the $CaCO_3$ microparticles coated with biocide against biofilms formed under flowing conditions using a flow cell reactor. Finally, Chapter 7, summarises the main findings of this work.

1.3. References

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Chapter 2

State of the art

Advances in the nanotechnology science led to its environmental and biological applications. Nanotechnology permits to create artificial systems with enormous potential for numerous applications. The aim here, in a biofilm control context, is the development of micro- nanostructures with functionalized surfaces that carry the antimicrobial agent. This will save significant amounts of the antimicrobial agent, prevent unwanted reactions with other components that can lead sometimes to the formation of undesirable disinfection by-products, and finally will reduce the costs of wastewater treatment due to the presence of residual concentrations of biocides in the effluent. Several methodologies to develop functionalized micro and nanoparticles are available, such as the layer-by-layer method, which represent a significant advance, in terms of surface science applications to the field of biofilm science and engineering. However, the application of such approaches for biofilm control has been limited, probably due to the lack of teams with expertise on both biofilm science and engineering and surface physicochemistry.

2.1. Biofilm formation and relevance

Biofilms can form on solid or liquid abiotic surfaces as well on soft tissue in living organisms and are typically resistant to conventional methods of disinfections (Huang et al., 2009) These biofilms are predominantly composed of bacterial cells enclosed within an extracellular polymeric (EPS) matrix which they produce (Davies et al., 1998; Decho, 2010). The EPS matrix is responsible for the cohesion (keeping the cells attached to one another) and adhesion (to surfaces) of the biofilm and is composed essentially of polysaccharides and proteins (Simões et al., 2010). Biofilms are ubiquitous in nature and its formation is a strategy that bacteria and other microbes use in order to survive in hostile environments.

Biofilms start to develop when bacteria or other microorganism attach to a surface. The polymers produced by the microbial cells play a determinant role in the adhesion process, sometimes creating a "polymer bridge" between the cells and the molecules initially adsorbed at the surface that strengthens the attached layer. The main processes involved in the biofilm build-up are: formation of a conditioning film; transport of the microorganisms from the bulk liquid to adhesion surface; initial adhesion of microorganisms to the surface; biofilm growth; biofilm maturation (equilibrium between the accumulation and detachment) (Melo, 2003; Simões et al., 2010) (Figure 2.1). The conditioning film is the first stage, preceding the formation of the bacterial film. The next stage is microbial reversible adhesion. At this stage nonspecific physicochemical force of interaction act between the molecules and microorganisms and the surface like: van der Waals forces, hydrophobic, electrostatic and London dispersion forces. Then irreversible adhesion begins when the cells become strongly attached to surface and to each other. Afterwards, the microorganisms start to excrete the extracellular polymers which form the EPS matrix (Nikolaev and Plakunov, 2007). Under favourable conditions, biofilm growth continues for a long time with loss of some cells and release of aggregates (detachment).

Some factors affect the formation and properties of biofilms, such as: characteristics of the microbial species and strains; composition and roughness of the surface material where the microorganisms are attached; nutrient composition and concentration, fluid pH, temperature and ionic strength; hydrodynamics of the fluid (velocity and turbulence) (Melo, 2003).
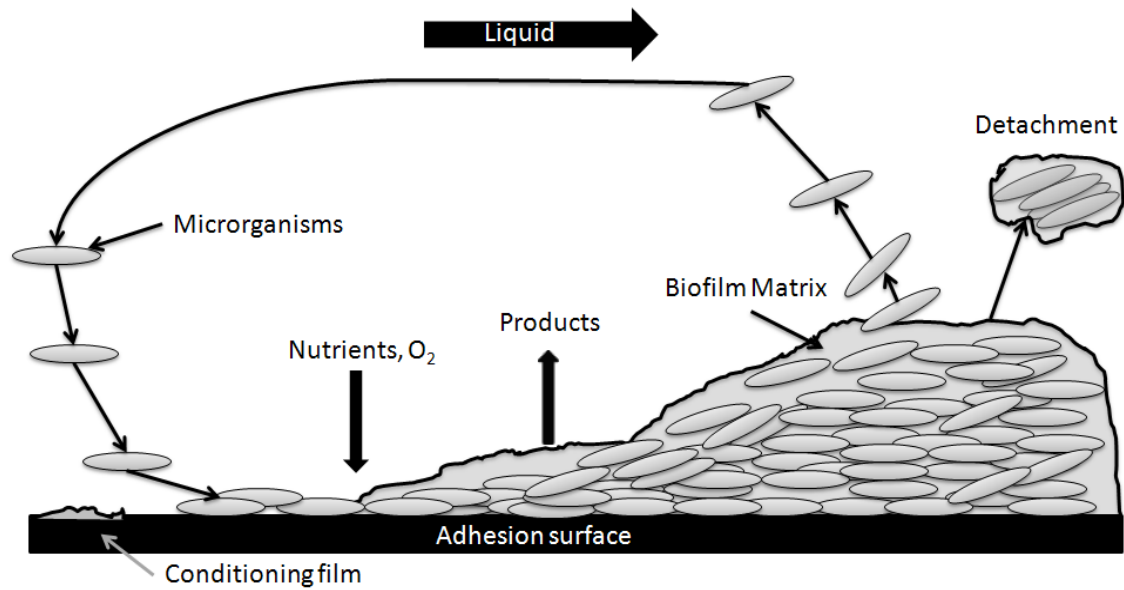
Figure 2.1. Schematic representation of biofilm formation process (adapted from Melo (2003)).

Despite all the efforts and advances in science, many health problems are still consequence of the lack of fresh and clean water. Microbial water pollution is a major problem both in industrialized and developing countries, with waterborne diseases (such as intestinal infections and diarrhea) remaining a significant cause of death (Li et al., 2008). Biofilms represent one major water contaminant as the majority of the microorganisms are comprised within these structures (Manuel et al., 2010). Biofouling also affects energy and food production as well as the biomedical, environmental, industrial and economic fields (Shannon et al., 2008). From the economic and environmental point of view, three of the most important examples of industrial biofouling occur in membrane systems, cooling water tubes and heat exchanger channels (Ferreira et al., 2010; Melo and Flemming, 2010). Biofilms can cause reduction of heat transfer, increased pressure drop, increased corrosion of metallic surfaces, contamination of the fluids flowing through the tubes and channels and of potable water produced in membrane systems, increased environmental costs associated to the need to treat wastewater containing the chemicals not consumed by the interaction with the microorganisms and increased costs of equipment and reagents for cleaning and disinfection (Melo and Flemming, 2010).

Although biofilms play crucial beneficial roles in many processes (like the biodegradation of environmental pollutants or the microbial balances within a body),

they often are unwanted and cause serious problems in various areas, including the industrial and biomedical fields, which can lead to increase in the costs of production and maintenance, as well as to public health and environmental concerns and impacts. This results in an increased use of antimicrobial chemicals, such as biocides, to control the unwanted formation of biofilms. In order to maximize the yield of action of biocides and to design efficient antimicrobials there is a growing need to comprehend not only the mechanisms of action of biocides, but also the mechanisms of resistance of bacterial cells (Russell, 2002).

2.2. Strategies for biofilm control

In industry, cleaning and disinfection are essential operations in the production process and the efficiency with which these operations are performed greatly affects the final product quality (Carpentier and Cerf, 1993). 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 contaminant residues by decreasing surface tension, emulsifying fats, and denaturing proteins (Maukonen et al., 2003). These chemical agents are currently used in combination. Many situations require the occasional use of acid cleaners to clean surfaces soiled with precipitated minerals or having high mineral content. Mechanical action (water turbulence and scrubbing) is recognized as being highly effective in eliminating biofilms (Chmielewski and Frank, 2003; Maukonen et al., 2003). An effective cleaning procedure must break up or dissolve the extracellular polymeric matrix associated with the biofilm so that disinfectants can gain access to the viable cells (Carpentier and Cerf, 1993; Gibson et al., 1999). The cleaning process can remove 90% or more of biofilm mass attached to the surface associated with the surface, but cannot be relied upon to kill the microorganisms. 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; Srinivasan et al., 1995).

Disinfection is the use of antimicrobials chemicals to destroy microorganisms. This 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 (Carpentier and Cerf, 1993; Holah, 1992). 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 (Cloete et al., 1998; Mosteller and Bishop, 1993). In food and drinking water processes, 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. Table 2.1 resumes the properties of disinfectants commonly used in industrial systems. The disinfectants to be used should be chosen based on the following statements (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?

A full strategy to control biofilms should include a toolbox of eco-friendly preventive methodologies that includes new antimicrobial agent techniques, innovation in equipment and process design and development of efficient and reliable on-line monitoring techniques (Pereira and Melo, 2009; Valladares Linares et al., 2015).

Table 2.1. Chemical disinfectants commonly used in industry (based on Banner (1995) and Wirtanen (1995))

Disinfectant type	Applications
Chlorine	Neutral/alkaline conditions; stainless steel; food contact surfaces; floors/walls/air; clean-in-place (CIP), spray, soak, fog
Chlorine dioxide	Water treatment/slime/odour control; rinse for fruit/vegetables; acid form on food contact surfaces; stainless steel; CIP, spray, soak
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
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
Peracetic acid	Acid conditions; carbon dioxide atmosphere; stainless and mild steel, soft metals, plastic, rubber; food contact surfaces; CIP, spray, soak
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
Amphoteric surfactants	Neutral/alkaline conditions; applicable to all materials; food contact surfaces; environmental areas; spray, manual soak; fog air; foam is suitable for external surface disinfection
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
Glutaraldehyde	Neutral/alkaline conditions; non-corrosive to all materials; water treatment/slime control in can/bottle warmers, tunnel pasteurizers; glycol and freshwater systems in dairies; conveyor lines
Isothiazolinones	Acid, alkaline, neutral conditions; applicable to all materials; cooling water/towers, can/bottle warmers; long-term, continuous activity; conveyor lubricants
Phenolics	Lubricants for conveyor lines; water treatment
Hydrogen peroxide	Applicable to all materials; sporicide at high concentration and high temperature; aseptic packing of beverages

2.3. Mechanisms of action of biocides

The word biocide is a general term to describe a chemical agent with antiseptic, disinfectant or preservative activity, that inactivates microorganisms (Russell, 2003). Some are capable of destroying the microorganisms (e.g. bactericidal and fungicidal) whilst others can only prevent or inhibit their growth (e.g. bacteriostatic and fungistatic) (McDonnell and Russell, 1999). Biocides have a broad spectrum of usage and differ from antibiotics in their lack of selective toxicity (Denyer and Stewart, 1998; McDonnell and Russell, 1999). In fact, they have multiple biochemical targets and have been used over the years in a diversity of areas (Simões et al., 2007). In medicine, biocides are used to prevent or limit microbial infections, preoperative skin disinfection or surface disinfection and in industrial systems they are used to prevent biofouling. But there are a lot more applications for these products. As examples, they are essential in food, pharmaceutical and cosmetic industries to prevent microbial contaminations (Masaadeh and Jaran, 2009; Russell, 2003). For their further design and development, it is vital to understand its mechanism of action as well as the mechanisms of resistance of bacteria.

Despite the final effect of biocides, they all undergo a sequenced series of steps: uptake of biocide by the cell, biocide partition to targets and its concentration at those targets and finally the damage of the targets (Denyer, 1995). The mechanism of action of these products can be divided into four wide categories: the oxidants (that act via radical-mediated reactions eliciting the oxidation of organic material), the electrophilics (that will covalently react with cellular nucleophiles thus inactivating some enzymes), the weak acids (that will interfere with the cell membrane ability to maintain the pH balance leading to the failure of the cell metabolism as a consequence of the acidification of the interior of the cell) and the cationic membrane active biocides (that weaken membranes as far as cell lysis) (Chapman, 2003).

Within bacterial cells, the biocides principal targets are the cell wall, cytoplasmic membrane and cytoplasm (Denyer, 1990). The main target is arguably the cytoplasmic membrane. Cell wall (composed by a network of peptidoglycan with a lipopolysaccharide overlayer in Gram-negative bacteria) (Figure 2.2) little more provides other than structural integrity and although there are a large number of molecules that allow cells to grow and multiply themselves (such as ribosomes, enzymes and other proteins) this molecules are probably not primary targets since biocides would have to penetrate inside the cell to reach them (Denyer, 1990; Maillard, 2002). Thus, the

cytoplasmic membrane is considered the main target of biocides. This membrane is composed by phospholipids forming a bilayer and some proteins embedded in this bilayer sheet, and it regulates the passage of solutes and metabolites in and out the cytoplasm (Maillard, 2002). Interaction of the biocide with the cytoplasmic membrane often causes changes both in membrane structure and function manifested by phenomena like disruption of the membrane, dissipation of the proton motive force, inhibition of the respiration reactions and membrane-associated enzymes activity and loss of membrane integrity with consequent leakage of essential intracellular constituents (Denyer, 1990; Denyer and Stewart, 1998; Maillard, 2002) , to name a few.
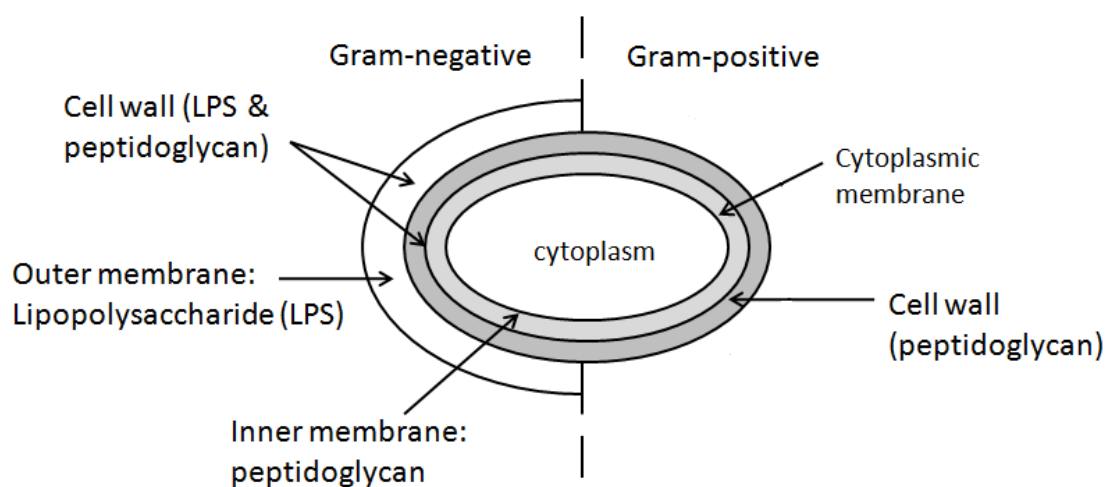
Figure 2.2. Bacterial cell wall composition (adapted from Denyer (1995)).

2.4. Bacterial mechanism of resistance

Biofilm resistance to biocides is increasing thus becoming a highly sensitive problem in industry and public health. Often, resistant bacteria are difficult to eradicate or even to treat. This brings a huge economic and environmental implication. In medicine, resistant bacteria are becoming frequent in hospitals and other healthcare institutions which can have serious consequences in patients, especially critically ill ones (Tenover, 2006). But the extension of resistant bacteria to other areas can have adverse implications in processes like cooling water systems, drinking-water distribution systems and food industries (Cloete, 2003). Some explanations for bacterial resistance include the limited penetration of antimicrobial compounds due to the presence of the EPS matrix, the imposition of nutrient deficiency in deeper biofilm bacteria, the transformation of the antimicrobial products into a non-toxic form by enzymes localized

in the biofilm matrix and the expulsion of biocide by the biofilm physical barrier to penetration preventing antimicrobials to reach its target(s) by size exclusion. Moreover, due to the charged nature of the matrix, diffusion of antimicrobial though the biofilm can be limited by chemical (ionic) interactions between biofilm and the matrix components that will act as an ion-exchange resin (Cloete, 2003; Gilbert et al., 2003; Simões et al., 2010). Within biofilms not all the cells receive the same amount of nutrients. Deep-lying cells are exposed to lower concentrations of substrates than outlying ones. This nutrient limitation will reduce the growth rates. Therefore biofilm cells will exist in a slow-growing state and in different metabolic states, making them less susceptible to antimicrobials (Costerton et al., 1999; Gilbert et al., 2002).

As mentioned before, bacterial genetic adaptation is another form of biofilm resistance to biocide. Bacteria may become resistant to antimicrobials by spontaneous mutation or by acquiring the genetic information for resistance from other bacteria. Mutations in the genetic information can translate in modification of the antimicrobial product target protein, production of specific enzymes that will inactivate the biocide, alteration of outer membrane protein channel required for the antimicrobial entry in cells or expression of efflux pumps (Tenover, 2006) that will extrude toxic substances for the bacteria (including therapeutic drugs) out of the cell. DNA and genetic technology allowed the identification of many genes in bacteria that confer resistance to biocides (Webber and Piddock, 2003).

Overall, Gram-negative bacteria such as *Escherichia coli* and *Pseudomonas fluorescens* are more resistant to biocides than the Gram-positive ones and this difference in susceptibility has been attributed to the fact that the Gram-negative bacteria possess an additional outer membrane (OM) that acts as an extra permeability barrier (McDonnell and Russell, 1999; Morton et al., 1998; Russell, 1999). The presence of lipopolysaccharide (LPS) molecules in the outer leaflet prevents the access of hydrophilic molecules (including antibiotics and biocides) to the cell interior (McDonnell and Russell, 1999; Morton et al., 1998). Moreover, in this OM there are a wide number of proteins (outer membrane proteins- OMP) that, although responsible for the entrance of hydrophobic antimicrobials to the cell, are associated to bacterial resistance, given that development of resistance to these products is related not only to loss of some OMP but also to the over-expression of OMP involved in antimicrobial resistance (Masuda et al., 1995; Winder et al., 2000).

2.5. Micro and nanotechnology in biofilm control

The conventional methods hitherto applied for water disinfection and decontamination have been generally effective in the control of microbial pathogens. However, new problems are being associated to them. Besides requiring a considerable economic effort and expensive infrastructures (Shannon et al., 2008), the chemical disinfectants are responsible for the production of harmful disinfection by-products (DBPs). Chemicals such as free chorine, chloramines and ozone can react with diverse natural water constituents thus forming DBPs, many of which are toxic and/or carcinogenic (Li et al., 2008; Shannon et al., 2008). For these reasons, and in order to successfully control waterborne pathogens in water, the development of new biofilm control strategies is imperative. Advances in the micro-nanotechnology field promoted significant interest in its environmental and biological applications.

Nanotechnology can be defined as the engineering and utilization of materials, structures, devices and systems at the atomic, molecular and macromolecular scale. Nanomaterials and nanostructures have nanoscale dimensions roughly between 1 and 100 nm and frequently exhibit novel physical, chemical and biological properties and functions resulting from their small structures (Roco, 2003; Theron et al., 2008). They are excellent adsorbents, catalysts, and sensors due to their large specific surface area and high reactivity (Li et al., 2008). Some of these nanomaterials are engineered to perform specific tasks, being able even to respond to outside signals by changing their structure and properties. For this, they are labelled as "smart" materials (Ratner and Ratner, 2003).

The field that applies this technology and structures to understand and transform biosystems can be defined as nanobiotechnology (Roco, 2003). Nanotechnology can present a unique alternative as detection methods of bacterial targets, in particularly nanosensors that could directly detect the presence of a particular pathogen agent or indirectly detect them through the assessment of the pathogens metabolic activity (e.g. by monitoring the nutrients consumption rate) (Neethirajan et al., 2014) (Kaittanis et al., 2008). Several engineered and natural nanomaterials have shown strong antimicrobial properties, including titanium dioxide, silver nanoparticles and chitosan. Some examples of the antimicrobial nanomaterials reported are chitosan, silver nanoparticles, photocatalystic TiO_2, to name a few (Li et al., 2008). Chitosan derives from chitin (a natural polysaccharide abundant in arthropod shells) and has been recently engineered

into nanoparticles (Qi et al., 2004). One of the antimicrobial mechanisms proposed for chitosan involves the interaction of positively charged chitosan molecules with negatively charged cell membranes, leading to an increase of membrane permeability and ultimately cell membrane rupture with consequent leakage of intracellular constituents (Li et al., 2008).

Metal nanoparticles such as palladium, platinum, silver and gold nanoparticles are very attractive due to their unique physical and chemical properties. In fact, gold nanoparticles have been widely used to construct biosensors because of their ability to immobilize biomolecules (Du et al., 2007). TiO_2 in nano scale is another material with antimicrobial properties. Due to its stability, low-cost and non-toxicity, is one of the most studied nanomaterials that is suitable for applications for example in water treatment (Hammond, 1999). Another promising approach is the use of carbon nanotubes for inhibiting microbial attachment and biofouling formation on surfaces. Carbon nanotubes are graphene sheets rolled into a tube which present antimicrobial activity against Gram-positive and Gram-negative bacteria and viruses (Lvov et al., 1993).

Nanoparticles present other advantages like high reactivity, unique interactions with biological systems, small size and large surface to volume ratio optimized for mass loading and carrying of antimicrobials (Taylor and Webster, 2009; Weir et al., 2008). Antimicrobials can be loaded into nanoparticles by physical encapsulation, adsorption or chemical conjugation and this can present several advantages such as significantly improve the activity of the antimicrobial, in contrast to the free product, and release of antimicrobial at a sustained and controlled manner (Zhang et al., 2008; Zhang et al., 2010). From several types of nanoparticles for antimicrobial delivery application, polymeric nanoparticles present the advantages of being structurally stable and of having on their surfaces functional groups that can easily be chemically modified with both antimicrobials and targeting ligands (Davis et al., 2008; Zhang et al., 2010).

Nanoscience and its application are very recent fields and fundamental properties of nanoparticles are being discovered every day. Further studies and investigation are still needed but the ability of nanoparticles to penetrate the biofilm, enter the cells and affect their biochemical functions makes them potential tools in biofilm control (Forier et al., 2014).

2.6. Layer-by-layer technique

The LbL self-assembly of oppositely charged polyelectrolytes onto colloidal particles has been used to create novel nano- and microparticles with well controlled size and shape, finely tuned wall thickness and variable wall compositions (Caruso et al., 1998; Cordeiro et al., 2004; Decher, 1997; Donath et al., 1998). The original method was introduced in 1991 by Decher and co-workers for the construction of pure polymer multilayer films on planar supports (Caruso, 2001).

This technique uses electrostatic attraction and complex formation between polyanions and polycations to form supramolecular multilayer assemblies of polyelectrolytes. The first stage of shell fabrication involves step-wise deposition of polyelectrolytes from aqueous solutions. The polyelectrolyte multilayer film is formed by the alternate adsorption of oppositely charged layers on to the particle. After each adsorption step, the non-adsorbed polyelectrolyte in solution is removed by repeated centrifugation or filtration and washing (Figure 2.3) (Donath et al., 1998).
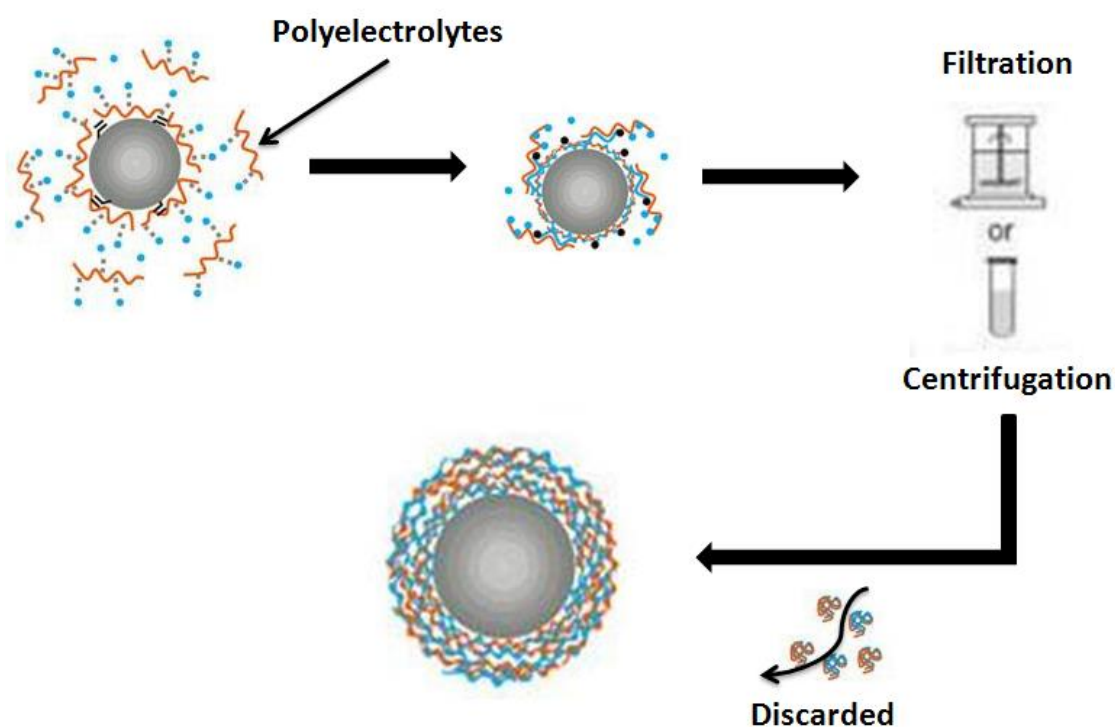
Figure 2.3. Schematic representation of LbL technique. Polyelectrolyte added into a system adsorbs onto the template leading to the charge reversal. After removal of polyelectrolyte excess (by washing of flat substrate or filtration or centrifugation of

colloidal cores), oppositely charged polyelectrolyte is added. The cycle is repeated to obtain a multilayer film or shell (adapted from Feng et al. (2007)).

Considering that each adsorption step leads to charge inversion of the surface, the subsequent deposition finally results in a layered complex, stabilised by strong electrostatic forces. For the construction of such self-assembled polyelectrolyte multilayers (PEMs) different building blocks have been used, including inorganic nanoparticles, functional polymers, proteins, chromophores, and biopolymers such as DNA (Hammond, 1999; Lvov et al., 1993). This method can be applied in the construction of planar layers, involving the use of different materials as a matrix for functional or biological molecular entities, e.g. for sensor applications, as separation membranes, or as tailored surface modification. The LbL assembly method presents various attractive advantages, including its simplicity and low cost and also the variability of the applicable materials. In addition to conventional polyelectrolytes, such as poly(allylamine hydrochloride) (PAH), poly(diallyldimethylammonium chloride) (PDDA), and poly(ethyleneimine) (PEI), poly(sodium styrenesulfonate) (PSS), various biomaterials have demonstrated to assemble by electrostatic LbL. This phenomenon occurs due to the presence of charged sites on their surface, leading to the formation of assembled films of proteins (Forier et al., 2014), DNA (Lvov et al., 1993) and charged polysaccharides. Charged inorganic substances, such as colloidal nanoparticles, clay, nanosheets, modified zeolite crystals, two-dimensional perovskite, and polyoxometalates have also been used for LbL assembly (Ariga et al., 2011).

A pioneer innovation in the LbL assembly reported in 1998 involves the application of the technique onto a colloidal particle core (Caruso et al., 1998). In other words, the LbL films were assembled sequentially, similarly to the conventional assemblies on a colloidal core.

Despite the simplicity associated to the procedures of the LbL strategy, the assembling mechanism stills not completely understood. According to experienced LbL researchers film structures and qualities depend significantly on experimental conditions (salt concentration, secondary interactions, temperature, etc.) (Schönhoff, 2003). Furthermore, deposition of materials in the LbL assembly is not always uniform. Although the LbL technology remains under development, the method already assures fabrication of nanostructures with positioning of target materials in desired geometries. The step-wise formation of these structures allows the introduction of multiple

functionalities, providing opportunities to engineer a new class of materials with unprecedented structure and function (Schönhoff, 2003). Furthermore, the surfaces can be modified to alter the functionality and improve the colloidal stability of the structure (Hammond, 1999). The easiness associated to this technique has been stimulating researchers in various fundamental fields including chemistry, physics, and biology, attracting them to work in practical applications including nanotechnology and materials technology (Ariga et al., 2007).

At present, there are two general approaches to encapsulate macromolecules into polyelectrolyte capsules using the LbL technique. The first method consists on the formation of particles out of molecules subjected to encapsulation. Dyes and drug nanocrystals were used to template LbL assembly leading to encapsulation. The second approach for encapsulation of macromolecules exploits preformed hollow capsules and incorporates the macromolecules from the surrounding medium by switching the permeability of the hollow capsule shell (Volodkin et al., 2004).

The environmental aspects of the current use of antimicrobials are of severe concern because of their residual presence in surface and ground waters and the consequent propagation to the food-chain, with risks to the public health. The controlled application and reuse of antimicrobials based on highly efficient strategies might avoid the dissemination of antimicrobial resistance. The prolonged exposure of microorganisms to sub-lethal concentrations promotes antimicrobial resistance and cross-resistance events (McDonnell and Russell, 1999; White and McDermott, 2001). There are several reports indicating the ability of bacteria to acquire resistance to quaternary ammonium compounds (Ishikawa et al., 2002; Méchin et al., 1999). Many studies have demonstrated that bacteria are capable of adapting to disinfectants used in industrial settings after prolonged exposure to sublethal concentrations (Aase et al., 2000; To et al., 2002). Loughlin et al. (2002) reported the ability of *P. aeruginosa* to adapt to increasing concentrations of benzalkonium chloride and verified the co-resistance to other membrane-active biocides. Also, the economical costs associated with the continuous application of antimicrobial chemicals should be considered.

2.7. References

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Chapter 3

Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on *Pseudomonas fluorescens*

Objective: Antimicrobial resistance is a major public health concern, particularly in hospitals and other health care settings. For the rational design of disinfection strategies it is of the utmost importance to understand the mechanisms of action of antimicrobials. In this study, the mechanism of action of benzyldimethyldodecylammonium chloride (BDMDAC) was assessed against *Pseudomonas fluorescens*.

3.1. Introduction

Control of microbial growth is required in many microbiologically sensitive environments, particularly when wet surfaces provide favourable conditions for microbial proliferation (Moore et al., 2008; Simões et al., 2010). An effective and wide spectrum disinfection strategy helps to prevent potential cross-resistance (that has so far been demonstrated *in vitro*) and the formation of resistant biofilms (Gilbert et al., 2002; Gilbert et al., 2003; Simões et al., 2008; Tattawasart et al., 1999). The word biocide is a general term to describe a chemical agent with antiseptic, disinfectant or preservative activity, that inactivates microorganisms (Russell, 2003). Some are capable of destroying the microorganisms (e.g. bactericidal and fungicidal) whilst others can only prevent or inhibit their growth (e.g. bacteriostatic and fungistatic) (McDonnell and Russell, 1999). Biocides have a broad spectrum of usage and differ from antibiotics in their lack of selective toxicity (Denyer and Stewart, 1998; McDonnell and Russell, 1999). In fact, they have multiple biochemical targets and have been used over the years in a diversity of areas (Simões et al., 2007). In health care environment, biocides are used extensively mainly for the disinfection of surfaces, water, equipment, and antisepsis, but also for the sterilization of medical devices and preservation of pharmaceutical and medicinal products (Maillard, 2005). Biocides are also used in industrial systems to prevent and control microbial growth and biofouling (Masaadeh and Jaran, 2009; Russell, 2003). For the design and development of effective antimicrobial strategies, it is vital to understand the mechanisms of action of biocides as well as the mechanisms of bacterial resistance.

Quaternary ammonium compounds (QACs) possess strong bactericidal, but weak detergent properties (McDonnell and Russell, 1999). Their antimicrobial properties depend on their structure and size, but especially on the length of the long-chain alkyl group. The structural functionality of QACs, especially the role of chain length on activity against different bacteria, has been previously observed (Tomlinson et al., 1977). The efficacy of QACs increases with temperature and pH (Paulus, 1993). They have a wide application ranging from the clinical to industrial purposes, particularly the disinfection of surfaces, equipments, medical devices and the preservation of products (Hugo and Russell, 1982; McDonnell and Russell, 1999; Paulus, 1993; Rossmoore, 1995).

The biocide BDMDAC used in this work is a QAC and a component of benzalkonium chloride (BAC). BAC is manufactured from coconut oil, which results in mixture of three alkyldimethylbenzlylammonium chlorides which differ only in the length of the alkyl side chains (C_{12}, C_{14} or C_{16}) (Bull et al., 1998; Ferrer and Furlong, 2002). BAC is widely used as disinfectant and sanitizer in hospitals, food plants, homes, and many public places (ordinary sanitation of noncritical surfaces, such as floors, furniture, and walls) (Takeoka et al., 2005). Its application includes preservatives and antiseptics in health care products (injectable solutions, eyewashes, nasal sprays, hand and face washes, mouthwashes, spermicidal creams, disinfection sprays) and as antistatic, emulsifiers and preservatives in coatings industry (paints, wood treatments and electronics) (Davis et al., 2010; Debreceni et al., 2007; Takeoka et al., 2005). All three components of BAC are surfactants with two regions in their molecular structure: a hydrocarbon water-repellent (hydrophobic) group and a water-attracting group (hydrophilic or polar). They can be classified as cationic, anionic, non-ionic and ampholytic, depending on the nature of their hydrophilic group (McDonnell and Russell, 1999). BDMDAC is cationic and has a long carbon chain composed of 12 carbons.

The purpose of this study was to investigate, using different physiological indices, the mechanisms of antimicrobial action of the QAC BDMDAC against the Gram-negative bacterium *Pseudomonas fluorescens*. *P. fluorescens* is an unusual cause of disease in humans, and usually affects patients with compromised immune systems (Gershman et al., 2008). This bacterium demonstrates hemolytic activity and, as a result, has been known to infect blood transfusions (Gibb et al., 1995). *P. fluorescens* already demonstrated the potential to form disinfectant-resistant biofilms and the ability to express resistant proteins when exposed to a QAC (Simões et al., 2006; Simões et al., 2008). In this study, *P. fluorescens* was chosen as a model bacterium to better understand the BDMDAC-bacteria interactions. This knowledge and understanding of the specific interactions between antimicrobials and bacterial cells offers a powerful tool in the search of improved biocidal capabilities. Choosing a disinfectant with an appropriate efficacy spectrum, in order to avoid problems with intrinsic resistance of microorganisms, is the first step in creating successful disinfection plans (Meyer and Cookson, 2010).

3.2. Material and methods

3.2.1. Microorganism

P. fluorescens was isolated from a drinking water distribution system and identified by 16S ribosomal DNA sequence analysis. The optimal growth conditions were 27 ± 3 ºC, pH 7, and glucose as the main carbon source. The *P. fluorescens* strain was cryopreserved in a refrigerated chamber at -80 ºC, in a mixture of nutrient broth and 30% (v/v) of glycerol. Bacteria propagation was obtained by removing an inoculum from the cryovial. The bacteria were then distributed evenly over the surface of Plate Count Agar (PCA – Merck, VWR) and incubated for 24 h at 27 ± 3 ºC.

3.2.2. Antimicrobial product

BDMDAC (Figure 3.1) was obtained from Fluka (Portugal). BDMDAC solutions were prepared from a stock solution (1 g/L) with sterile distilled, before each experiment.
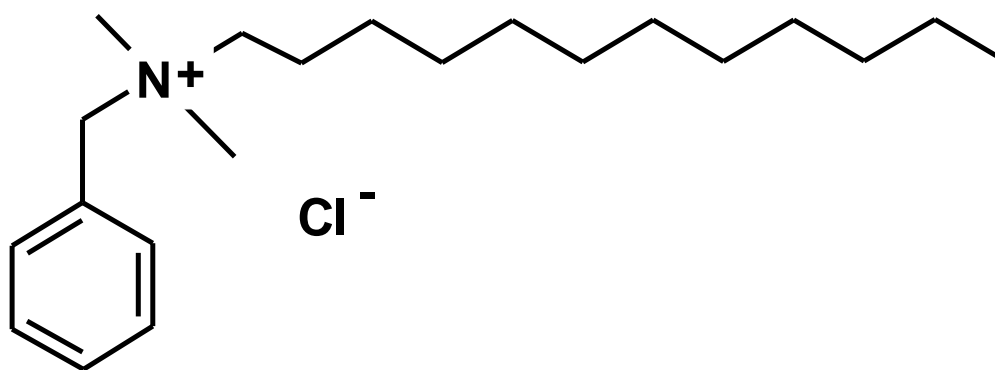
Figure 3.1. Chemical structure of BDMDAC

3.2.3. Growth inhibitory activity and minimum inhibitory concentration

Bacterial cultures were prepared in 200 mL sterile flasks, containing 50 mL of sterile growth medium (5 g/L glucose, 2.5 g/L peptone and 1.25 g/L yeast extract in 0.02 M phosphate buffer) and an appropriate volume of bacterial inoculum. The optical density, at 610 nm (OD_{610}), was set to 0.2 ± 0.02, corresponding approximately to

1.5×10^8 cells/mL. In each of these bacterial cultures, a different concentration of BDMDAC (0, 5, 10, 15 and 20, and 40 mg/L) was established, followed by incubation in an orbital shaker (120 rpm, 27 ℃). Bacterial growth was measured at specific time points (between 0 and 24 h) by aseptically sampling 1 mL from each flask and subsequently measuring the OD_{610} (T80 UV/Vis spectrometer, PG Instruments Ltd). These growth assays were performed in triplicate.

To determine the effects of BDMDAC on bacteria ability to grow in a liquid culture, the minimum inhibitory concentration (MIC) was assessed, using the macrobroth dilution method (Champlin et al., 2005). The MIC was determined as the lowest concentration of biocide, where no growth was detected (Gilbert and McBain, 2003).

3.2.4. Minimum bactericidal concentration

An overnight grown culture was centrifuged (3220 g, 15 min) and washed two times with saline solution (0.85% NaCl). Afterwards, bacteria were resuspended in saline solution to obtain an OD_{610nm} of approximately 0.2 ± 0.02. Then an aliquot of 1 mL of this suspension was collected and different concentrations of biocide were tested (0, 1, 2, 3, 4, 5, 7.5, 10, 15, 20, 40, 60, 80, 100 mg/L). After 60 min of contact the suspensions were subjected to a process of biocide neutralization, according to Johnston et al. (2002). BDMDAC was chemically neutralized (10 min contact time) by the following solution: (w/v) 0.1 % peptone (Sigma; Portugal), 0.5 % Tween 80 (Sigma) and 0.07 % lecithin (Sigma), dissolved in saline solution. Afterwards, bacterial suspensions were diluted to an adequate cellular concentration (from 10^5 to 10^0) in saline solution. Then, a volume of 100 μL of each suspension was transferred onto PCA plates and incubated at 27 ± 3 ℃. Colony enumeration was carried out after 24 h. The lowest concentration of BDMDAC where no colony forming units (CFU) were detected on solid medium was taken as the minimum bactericidal concentration (MBC) (Smith et al., 2008). Three independent experiments were performed for each condition tested.

3.2.5. Assessment of membrane integrity due to propidium iodide uptake

The Live/Dead *Bac*Light[TM] kit (Invitrogen) assesses membrane integrity by selective stain exclusion (Simões et al., 2005). This fast method was applied to estimate

both viable and total counts of bacteria. *Bac*Light is composed of two nucleic acid-binding stains: SYTO 9[TM] and propidium iodide (PI). SYTO 9[TM] penetrates bacterial membranes, staining the cells green; PI only penetrates cells with damaged membranes, binding to single- and double-stranded nucleic acids. The combination of these two stains generates red fluorescing cells. After antimicrobial treatment (60 min), the suspensions were subjected to a process of biocide neutralization, as described previously. Afterwards, bacteria were transferred to saline solution and diluted 1:10. Three hundred microliters of each diluted suspension were filtered through a Nucleopore® (Whatman) black polycarbonate membrane (pore size 0.22 μm) and stained with 250 μL diluted SYTO 9 [TM] and 250 μL diluted component PI. The dyes were left to react for 15 min in the dark, at 27 ± 3 ℃. The membrane was then mounted on *Bac*Light mounting oil, as described in the instructions provided by the manufacturer. The microscope use for observation of stained bacteria was a LEICA DMLB2 with a mercury lamp HBO/100W/3 incorporating a CCD camera to acquire images using IM50 software (LEICA) and a 100× oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained mounts consisted of a 480 to 500 nm excitation filter in combination with a 485 nm emission filter (Chroma 61000-V2 DAPI/FITC/TRITC). A program path (Scan Pro 5) involving object measurement and data output was used to obtain the total number of cells (both stains) and the number of PI stained cells. Both the total number of cells and the number of PI stained cells on each membrane was estimated from counts of a minimum of 20 fields of view. The range of total cells per field was between 50-200 cells. Three independent experiments were performed for each condition tested.

3.2.6. Physico-chemical characterization of bacterial surface

The physico-chemical properties of the bacterial surface were determined by the sessile drop contact angle measurement on bacterial lawns, prepared as described by Busscher et al (1984). Determination of contact angles was performed automatically using an OCA 15 Plus (DATAPHYSICS, Germany) video based optical contact angle measure instrument, allowing image acquisition and data analysis. Contact angle measurements (at least 25 per liquid and OPA concentration tested) were carried out according to Simões et al. (2007) (Figure 3.2). The liquids surface tension components

reference values were obtained from literature (Janczuk et al., 1993). Hydrophobicity was evaluated after contact angles measurements, following the van Oss approach (van Oss et al., 1987; van Oss et al., 1988; van Oss et al., 1989) where the degree of hydrophobicity of a given surface (s) is expressed as the free energy of interaction between two entities of that surface, when immersed in water (w) - ΔG_{sws} (mJ/m^2). If the interaction between the two entities is stronger than the interaction of each entity with water, $\Delta G_{sws} < 0$, the material is considered hydrophobic. Conversely, if $\Delta G_{sws} > 0$, the material is hydrophilic. ΔG_{sws} can be calculated through the surface tension components of the interacting entities, according to:

$$\Delta G_{sws} = -2\left(\sqrt{\gamma_s^{LW}} - \sqrt{\gamma_w^{LW}}\right)^2 + 4\left(\sqrt{\gamma_s^+ \gamma_w^-} + \sqrt{\gamma_s^- \gamma_w^+} - \sqrt{\gamma_s^+ \gamma_s^-} - \sqrt{\gamma_w^+ \gamma_w^-}\right) \tag{1}$$

where γ^{LW} accounts for the Lifshitz-van der Waals component of the surface free energy, and γ^+ and γ^- are, respectively, the electron acceptor and electron donor parameters, of the Lewis acid-base component (γ^{AB}), being $\gamma^{AB} = \sqrt{\gamma^+ \gamma^-}$.

The surface tension components, of a solid material, are obtained by measuring the contact angles of the three liquids (l), the apolar α-bromonaphtalene, and the polar formamide and water. All these three pure liquids hold well known surface tension components. Once the values are obtained, three equations of the type below can be solved:

$$(1 + \cos\theta)\gamma_l^{Tot} = 2\left(\sqrt{\gamma_s^{LW} \gamma_w^{LW}} + \sqrt{\gamma_s^+ \gamma_w^-} + \sqrt{\gamma_s^- \gamma_w^+}\right) \tag{2}$$

where θ is the contact angle and $\gamma^{TOT} = \gamma^{LW} + \gamma^{AB}$.

At least, three independent experiments were performed for each condition tested.

Figure 3.2. Apparatus model OCA 15 Plus (Dataphysics Instruments, Germany), for contact angle measurements after capturing of the drop image in the surface of bacterial lawns with a video camera.

3.2.7. Bacterial surface charge - zeta potential

The zeta potential of cells suspension before and after the contact with different BDMDAC concentrations (5, 10, 15, 20, 60 and 100 mg/L) was determined using a Nano Zetasizer (Malvern instruments, UK). Cells suspensions without biocide were used as control. The zeta potential was measured by applying an electric field across the bacterial suspensions. Bacteria in the aqueous dispersion with non-zero zeta potential migrated toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. The experiments were repeated at least at three different occasions.

3.2.8. Potassium (K^+) leakage

The flame emission and atomic absorption spectroscopy was used for the K^+ titration in solutions of *P. fluorescens* with different concentrations of BDMDAC. The samples were filtrated after contact with biocide and then were analyzed in a GBC AAS 932 plus device and the acquisition with GBC Avante 1.33 software. The samples were measured at three independent experiments.

3.2.9. Scanning electron microscopy

Prior to SEM observations, bacterial cells were dehydrated by heat (45 ºC, 3 h) and stored in a desiccator for 3 days. The samples were sputter-coated with gold and the SEM observations were performed with a JEOL JSM-6301F microscope coupled with the microanalysis system OXFORD INCA/ENERGY 350. The bacteria were observed before and after exposure to BDMDAC.

3.2.10. Statistical analysis

The data were analysed using the statistical program SPSS 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Because low samples numbers contributed to uneven variation, nonparametric Wilcoxon test procedure was used to compare the different conditions tested. Statistical calculations were based on confidence level equal or higher than 95% ($P < 0.05$ was considered statistically significant).

3.3. Results and discussion

P. fluorescens was chosen as a model system to better understand the interactions between BDMDAC and bacterial cells. This bacterium is a well-studied Gram-negative bacterium ubiquitous in nature, medical and industrial environments and has potential to cause serious problems in a wide range of areas in its planktonic and biofilm states (Hsueh et al., 1998; Simões et al., 2010; Tuttlebee et al., 2002). Although not so infectious as other *Pseudomonas* species (e.g. *P. aeruginosa*), *P. fluorescens* has been emerging as important nosocomial pathogens in cancer patients (Rolston et al., 1987) This bacterium has a strong ability to form disinfectant-resistant biofilms (Simões et al., 2008).

BDMDAC is a QAC with bacteriostatic or bactericidal properties depending on the concentration (Ferrer and Furlong, 2001). Although it appears to have surface-active properties, little is known about its mechanism of action. The present study provides additional information on BDMDAC mechanism of action against Gram-negative bacteria. The MIC of BDMDAC against *P. fluorescens* was found to be 20 mg/L (Figure 3.3). This value is lower than the concentrations of biocide that are commonly used in

some disinfection processes (approximately 50 mg/L) (Ferreira et al., 2010; Simões et al., 2007).

The analysis of bacterial growth in the presence of various concentrations of BDMDAC over time (Figure 3.3) show that at a concentration of 5 mg/L cells have the same growth behaviour ($P > 0.05$) as the control (cells without biocide). For concentrations of 10 and 15 mg/L the growth profile was different from the control ($P < 0.05$) and the cells start to grow only after few hours of adaptation (~ 8 h). For concentrations of 20 mg/L and higher, BDMDAC inhibits cell growth. Additional bacterial growth experiments were performed with BDMDAC at 60, 80 and 100 mg/L providing similar results to those obtained with 20 and 40 mg/L.
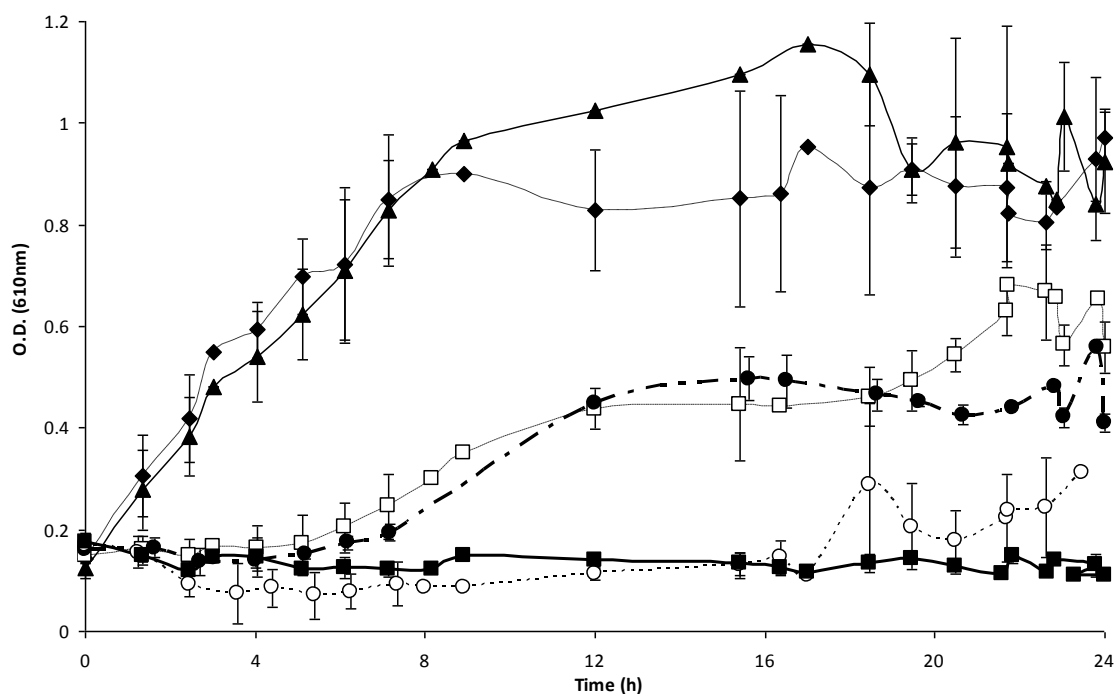
Figure 3.3. Growth curves of *P. fluorescens* over time for different concentrations of BDMDAC (◆ - control; ▲ - 5 mg/L; □ - 10 mg/L; ◕ - 15 mg/L; ◌ - 20 mg/L; ■ - 40 mg/L). The means ± SDs for at least three replicates are illustrated. The concentration with no visible growth was considered the MIC.

The MBC of BDMDAC was 10 mg/L (Figure 3.4). However, total (100% cells) membrane damage was obtained with BMDAC at 20 mg/L (Figure 3.5). The Live/Dead *Bac*Light viability kit allows the discrimination of viable and dead cells in a bacterial population. According to this method, those cells fluorescing green (without membrane

damage) are considered viable cells while those fluorescing red (with membrane damage) are considered dead cells (Figure 3.5 and Figure 3.6). There is no consistency ($P < 0.05$) between the CFU counts and the number of potential viable cells for concentrations of 0, 1, 2, 5, 10, 15 and 20 mg/L (Figure 3.4).
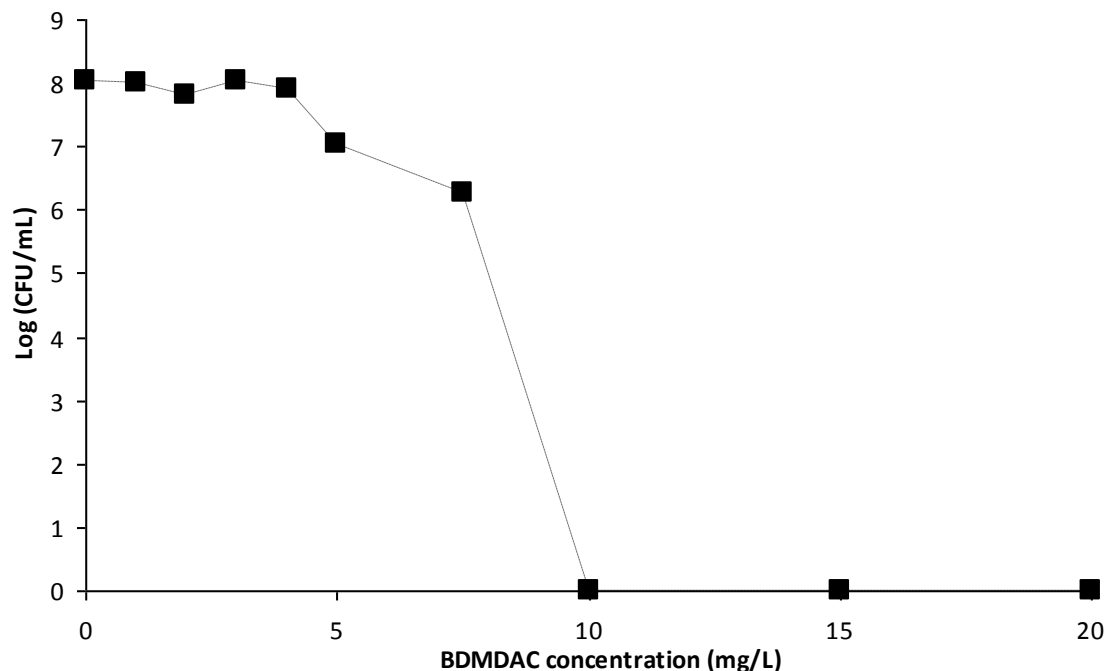
Figure 3.4. Log CFU counts of *P. fluorescens* as function of BDMDAC concentration (0, 1, 2, 3, 4, 5, 7.5, 10, 15 and 20 mg/L) after 1 h exposure period. The means ± SDs for at least three replicates are illustrated. Standard deviations are very small and therefore, cannot be distinguished in the Figure. The MBC value was determined as the lowest concentration where no CFU counts were detected.

This observation is most likely a consequence of the limitations of the plate count method. It has already been recognized by many authors that the quantification of CFU has several drawbacks (Banning et al., 2002; McFeters et al., 1995; Simões et al., 2005; Stewart et al., 1994). This technique can underestimate the numbers of viable cells due to, among others, the presence of injured or starved cells or potentially 'viable but nonculturable' cells (VBNC). Hence, alternative approaches like epifluorescence microscopy with viability-indicator stains provide good complementary information about the antimicrobial effects of biocides (Figure 3.4).
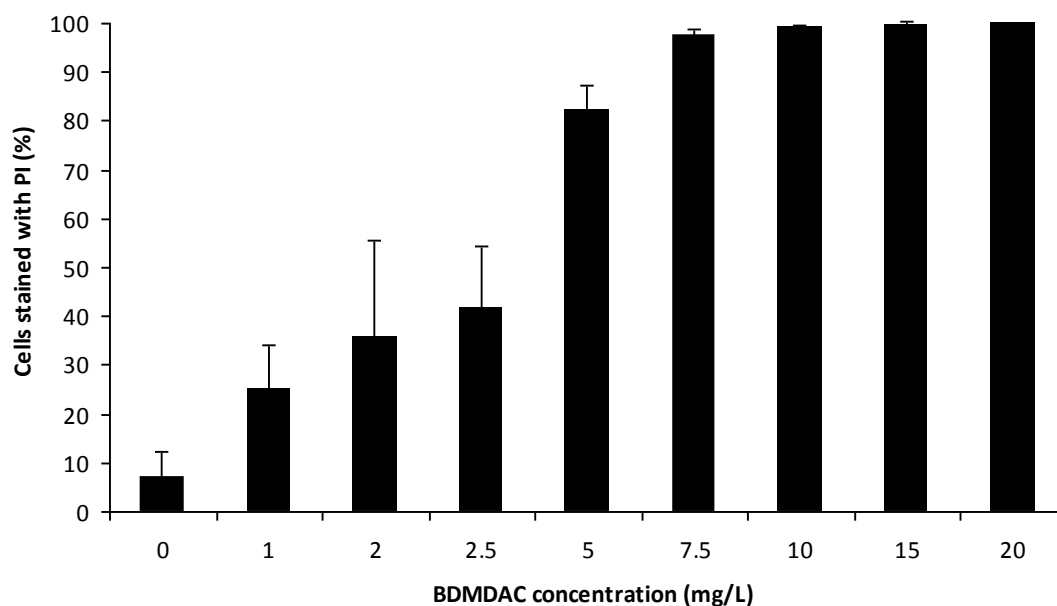
Figure 3.5. Permeability of *P. fluorescens* to PI after treatment with BDMDAC at different concentrations for 1 h. The percentage of cells non-stained with PI corresponds to the fraction of viable cells. The means ± SDs for least three replicates are illustrated.
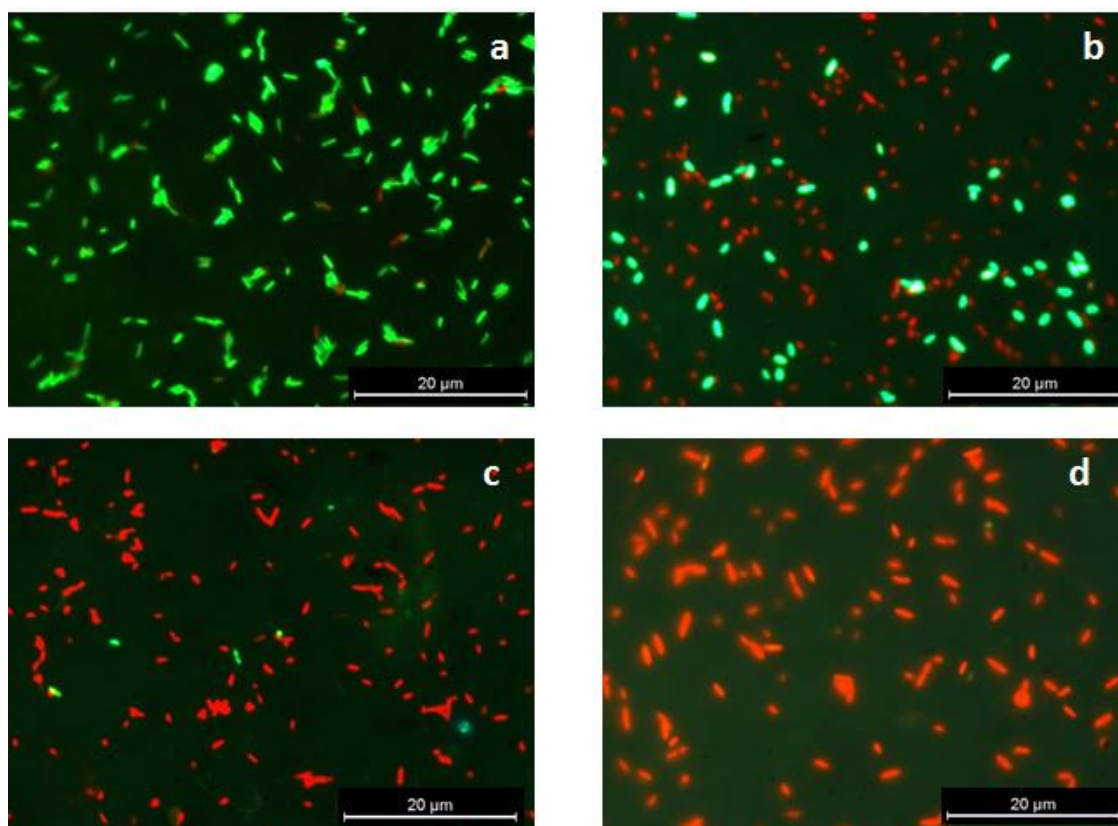
Figure 3.6. Epifluorescence photomicrographs of *P. fluorescens* ×1000. a) 0 mg/L BDMDAC, b) 5 mg/L BDMDAC, c) 10 mg/L BDMDAC and d) 20 mg/L BDMDAC.

The differences in the numbers of potential viable cells (cells without membrane damage), CFU and VBNC are highlighted in Figure 3.7. The number of VBNC cells is given by the difference between the number of viable cells and the number of CFU. Although VBNC lose their ability to grow on media typically used for culture, they can still alive and capable of resuscitation, regaining their metabolic activity, once the conditions are favorable (Oliver, 2005). Thus, it becomes imperative to combine several methodologies to provide more complete information about the action of biocides on bacteria.
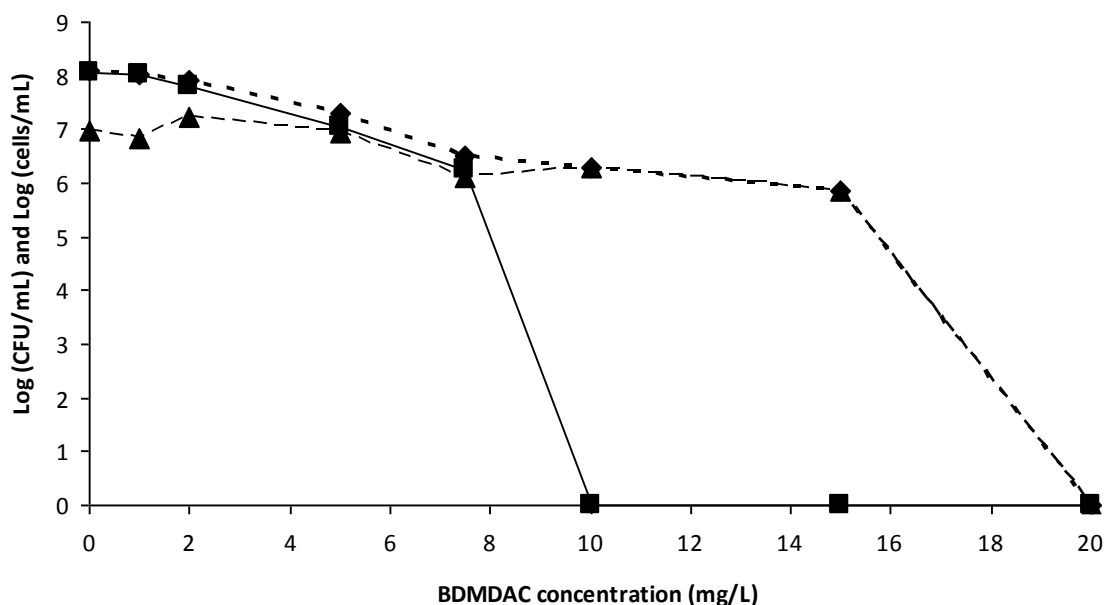
Figure 3.7. Number of viable cells (without membrane damage - ◆), CFU (■) and VBNC (▲) as a function of BDMDAC concentration, after 1 h exposure period. The means ± SDs for at least three replicates are illustrated. Standard deviations are very small and therefore, cannot be distinguished in the Figure.

Due to anionic groups, such as carboxyl and phosphate, present in its membrane, most microorganisms have a negative surface charge under physiological conditions (Ahimou et al., 2002). The charge properties of the cell surfaces play a role in bacterial resistance mechanisms to positively charged biocides such as QACs (Bruinsma et al., 2006). The surface charge of cells is often determined as its zeta potential, calculated from the mobility of cells in the presence of an electrical field under well-defined pH and salt concentrations (Palmer et al., 2007). The results obtained from the zeta potential assay (Figure 3.8a) allowed to better understand how the positively charged BDMDAC

interacts with the bacteria. *P. fluorescens* cells are negatively charged. However, the exposure to BDMDAC changes the cells surface charge to less negative values. In fact, it is possible to divide the results in two groups: for concentrations between 5 and 15 mg/L, the zeta potential values obtained are similar to the control ($P > 0.05$) and for concentrations above 20 mg/L the values were significantly different from the control ($P < 0.05$). This concentration and above seems to be critical for the damage of *P. fluorescens* cytoplasmic membrane (Figure 3.5).

The PI uptake results suggest that BDMDAC seriously compromise the integrity of the cytoplasmic membrane (Figure 3.5). A concentration of 1 mg/L is enough to damage the cytoplasmic membrane of 20% of the cell population. The percentage of cells with membrane damage increases considerably with BDMDAC concentration. With 5 mg/L the percentage of damage was already above 80 %. For concentrations above 15 mg/L all cells (100%) presented cytoplasmic membranes damaged, losing their viability.

Potassium (K^+) leakage is a good indicator of the cytoplasmic membrane damage (Codling et al., 2003; Lambert and Hammond, 1973). The amount of K^+ release from the bacterial cells is dependent on the biocide concentration, increasing with the increase of BDMDAC concentration ($P < 0.05$). This indicates an alteration in the cytoplasmic membrane permeability. For a concentration of 20 mg/L the amount of K^+ released was similar ($P > 0.05$) to the amount released for a concentration of 100 mg/L (Figure 3.8b). These results are strongly correlated with the change on cell surface charge (linear correlation: $R^2 = 0.937$). Moreover, the results obtained for the leakage of K^+ can be also correlated with the PI uptake results (linear correlation: $R^2 = 0.911$). This correlation is expected given that PI only penetrates cells that have lost cytoplasmic membrane integrity and if the number of cells with damaged membranes increases with the augmentation of the biocide concentration, the leakage of K^+ will also increase.
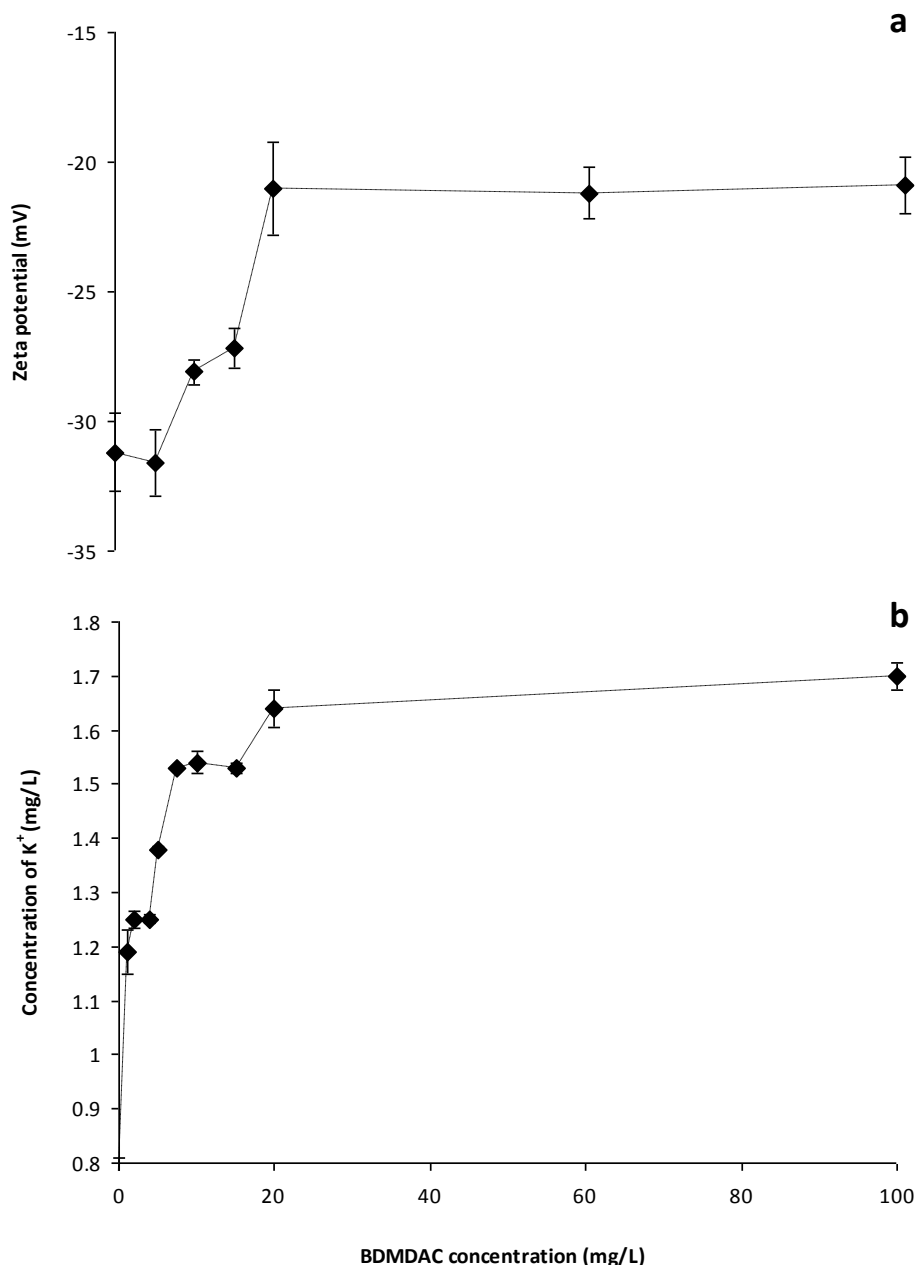
Figure 3.8. Values of the zeta potential (mV) of suspensions of *P. fluorescens* in contact with different concentrations (0, 5, 10, 15, 20, 60 and 100 mg/L) of BDMDAC for 1 h (a). K^+ concentration (mg/L) in solution after the contact of *P. fluorescens* with different concentrations (0, 1, 2, 4, 5, 7.5, 10, 15, 20 and 100 mg/L) of biocide for 1 h (b). The means ± SDs for at least three replicates are illustrated.

The results from the bacteria surface physicochemical characterization show that cell-BDMDAC interaction results in an alteration of cell surface hydrophobicity (Table 3.1). *P. fluorescens* cells present hydrophilic properties ($\Delta G^{TOT} > 0$ mJ/m^2). The application of BDMDAC promotes the decrease of their hydrophilic characteristics. The

values of the surface tension components demonstrated that the cells acquired polar properties after BDMDAC treatment, translated by the increase of the γ^{AB} values for the concentrations of 10 and 20 mg/L in comparison to control and to BDMDAC at 5 mg/L ($P < 0.05$). The apolar component γ^{LW} was almost unaffected by the BDMDAC treatment ($P > 0.05$). Several authors (Glover et al., 1999; Massi et al., 2003) have demonstrated that cytoplasmic membrane disturbance may not be immediately responsible for biocidal efficacy. According to Maillard (2002) one of the noticeable effects of biocidal interaction with bacterial cells is a change in hydrophobicity. In fact, in this study, the BDMDAC action resulted in a change of cellular hydrophobic interactions (Table 3.1), suggesting the possible existence of BDMDAC-membrane binding receptors. Such results might indeed be the cause of membrane function impairment. The physico-chemical alterations at cell surface level, and subsequent PI uptake, probably caused irreversible bactericidal activity. In Gram-negative bacteria, the passage across the outer membrane depends on the chemical nature of the antimicrobial agent, with hydrophilic agents utilizing the porin channels – hydrophilic route - and hydrophobic agents entering *via* the hydrophobic route. This occurs probably due to the disturbance of the lipidic fraction of the outer membrane, as the lipophilic nature of BDMDAC will play a key role in the diffusion through the outer membrane (Jarlier and Nikaido, 1990). In previous studies (Carson et al., 2002) where the mechanisms of action of lipophilic biocides have been examined, the effects on the cytoplasmic membrane, and/or on enzymes embedded in it, have been demonstrated. In this study, the data from contact angles assessment and the zeta potential values proposes that BDMDAC could exercise its effects through monolayer or supramonolayer adsorption at the cell surface. Other authors (Hugo and Longworth, 1966; Ioannou et al., 2007) previously described the ability of cationic biocides to form monolayers on the surface of bacterial cell. Hamilton (1968) previously reported that a common feature of QACs is their ability to cause cell leakage and membrane damage, primarily due to their adsorption to the bacterial membrane in large amounts. A 1-D electrophoresis assay was also performed in order to ascertain if there was a loss of outer membrane proteins (OMPs) or the over-expression of OMPs (resistance OMPs). However, no alteration in OMPs expression was detected, demonstrating that BDMDAC exerts antimicrobial activity without allowing the expression of resistance proteins at the outer membrane. In previous

studies, it was reported that cationic biocides seem to promote the expression of resistance OMPs (Masuda et al., 1995; Mitchell et al., 1998; Simões et al., 2006).

Table 3.1. Hydrophobicity (ΔG_{sws}^{TOT}), and apolar (γ^{LW}) and polar (γ^{AB}) components of the surface tension of untreated and BDMDAC treated cells. The means ± SDs for at least three replicates are illustrated

Concentration of BDMDAC (mg/L)	Surface tension parameters (mJ/m²)				ΔG^{TOT} (mJ/m²)
	γ^{LW}	γ^{AB}	γ^{+}	γ^{-}	
0	17.2 ± 0.3	11.5 ± 1.4	0.4 ± 0.1	74.6 ± 0.3	62.3 ± 1.6
5	17.9 ± 2.4	19.8 ± 6.5	1.7 ± 0.9	59.5 ± 4.4	36.5 ± 0.3
10	20.1 ± 0.7	32.4 ± 1.3	4.4 ± 0.3	59.5 ± 0.6	31.3 ± 1.5
20	23.3 ± 0.3	28.4 ± 1.4	3.8 ± 0.3	53 ± 0.4	27.6 ± 0.4

In order to detect structural modifications of *P. fluorescens* as a result of BDMDAC treatment, bacterial samples were analyzed by SEM. Cells treated with 20 mg/L (lethal concentration) are less bulky and their membrane seems to be rougher, wrinkled, deformed (Figure 3.9) compared with untreated cells. This is apparently a result of the high cell-BDMDAC interaction that, in addition to disrupt cell membranes, it promotes the release of intracellular material, changing significantly the cell homeostasis.
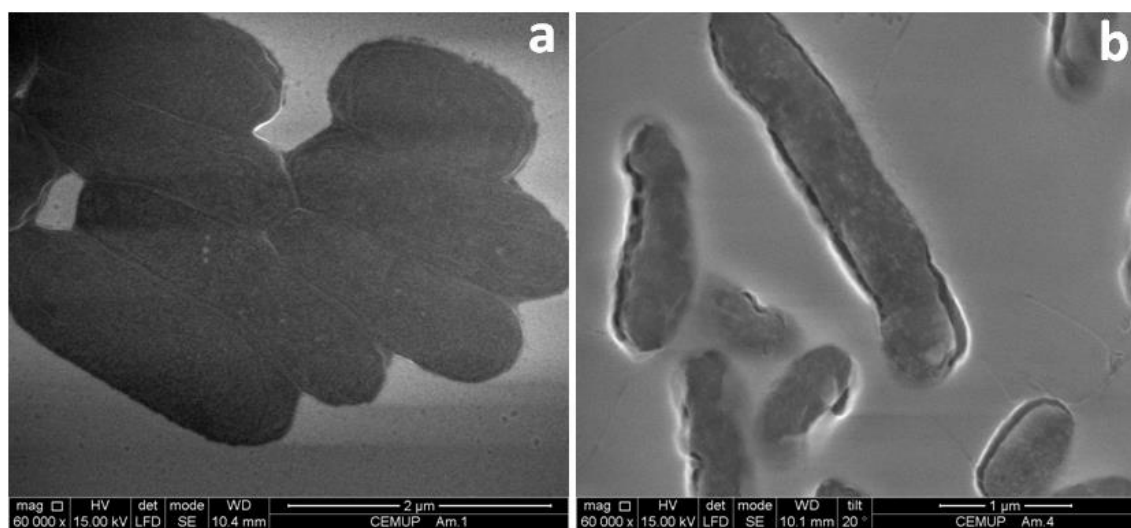
Figure 3.9. SEM photomicrographs of *P. fluorescens* before (a) and after (b) exposure to 20 mg/L of BDMDAC for 1 h.

In conclusion, a systematic approach was used to identify physiological aspect affected by BDMDAC activity, beginning with an initial focus on bactericidal activity followed by an assessment of cell membrane sensitivity and change in properties. *P. fluorescens* is susceptible to BDMDAC. The biocide has strong antimicrobial activity with MIC of 20 mg/L and MBC of 10 mg/L. It interacts strongly with cell surfaces in a concentration dependent manner, bind by ionic and hydrophobic interactions to microbial membrane surfaces, manifested by phenomena like disruption of the membrane and loss of membrane integrity with consequent leakage of essential intracellular constituents, promoting significant and irreversible changes in the cell structure. The overall bactericidal events occurred without detectable resistance phenomena such as the expression of resistant OMPs, suggesting that BDMDAC is an interesting biocide for *P. fluorescens* disinfection.

3.4. References

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Chapter 4

Biofilm control in a chemostat using polystyrene microparticles carrying a biocide

Objective: This study presents a new technological approach to minimize the use of antimicrobial agents and their deleterious effects, based on the principle of drug-delivery systems where the antimicrobial chemicals are transported on microparticles. The efficacy of microparticles carrying the benzyldimethyldodecylammonium chloride (BDMDAC*)* was assessed against *Pseudomonas fluorescens* in planktonic and biofilm states.

4.1. Introduction

Layers of microorganisms and their extracellular polymers (biofouling) grow very frequently on industrial cooling water tubes and heat exchanger channels, causing increased pressure drop and reduced heat transfer efficiency (Rao et al., 2009; Vrouwenvelder et al., 2009). These problems lead, ultimately, to an increase in the costs of production and maintenance, as well as to public health problems and environmental impacts (Pereira et al., 2008). Often, the layers build up in a non-uniform manner, with localized spots where thicker biofilms appear. To prevent and/or remove biofilms from surfaces, biocidal products and dispersants are introduced in the water stream in considerable large amounts. Such toxic chemicals are not totally consumed via the interaction with the biological structures and their discharge into the environment or wastewater treatment plants is a source of environmental and public health problems (Rasmussen et al., 1999).

Modern strategies to minimize biofouling in industrial equipment (pipes, heat exchangers, etc) have been focused on optimizing process conditions and equipment design, developing new surfaces to reduce adhesion and applying efficient surface cleaning/disinfection methodologies, supported by on-line monitoring techniques. A considerable amount of work has been reported on the effect of process conditions, such as hydrodynamics, on biofilm growth and process performance (Charaeklis, 1990; Melo and Vieira, 1999). Although further advances are still possible on this aspect, the improvements will be generally marginal. The use of liquid velocities around 2 m/s inside tubes is recommended in order to take advantage of the stronger shear stresses, reducing biofilm growth. However, high velocities have some drawbacks as they produce more compact deposits that are more difficult to remove from the surfaces by both mechanical and chemical methods (Melo and Vieira, 1999; Simões et al., 2005; Simões et al., 2008a) and higher corrosion rates. Several attempts have been made to avoid biofilm formation by the incorporation of antimicrobial products into surface materials (Park et al., 2004; Weng et al., 1999) or by coating surfaces with antimicrobials (Akesso et al., 2009; Chen et al., 2009; Gottenbos et al., 2002; Thouvenin et al., 2003). More recently, new low energy surfaces produced by surface bombardment to implant ions such as Mo and F, plasma sputtering and coatings with thin Ni-P-PTFE or DLC (Diamond-Like-Carbon) layers have been developed with an interesting potential for reducing deposit adhesion (Zhao et al., 2009). However, their application is much

dependent on the relative costs of such expensive materials comparatively to the costs associated with fouling (Rosmaninho and Melo, 2006; Rosmaninho et al., 2007) and their efficacy tends to decrease over time. Additionally, it was shown that the major advantage of some of these surfaces is that they promote the formation of deposits that are easier to clean (Rosmaninho et al., 2007). Therefore, cleaning has increasingly become the crucial step in the optimization of these systems. There are, here, two interconnected issues: a) biofilms and other deposits do not attach uniformly along the surfaces; b) chemicals used to remove biofilms are carried as solutes by the bulk liquid and only a minor fraction does actually take part in the cleaning process, leaving a large amount in the discharged waters. The current cleaning procedures are still highly inefficient processes, consuming large amounts of water, chemicals and time (Ludensky, 2003). More efficient cleaning techniques are needed in order to reduce the consumption of water, chemicals, energy and time, and, simultaneously, to efficiently control biofouling. This will reduce the use of toxic chemicals and will minimize health and environmental risks of antimicrobial chemicals (Gilbert and McBain, 2003).

The methodology proposed in the present work is based on the use of microparticles with functionalized surfaces that act as carriers of antimicrobial molecules. The biocide selected in this study benzyldimethyldodecylammonium chloride (BDMDAC) is commonly applied as a disinfectant in swimming pools (concentrations from 2 mg/L, in day-to-day water treatment, up to 8 mg/L for heavy algae growth) and in cooling water systems (concentrations of the order of 50 mg/L of active product) where disinfection is often achieved by recirculating the biocidal solution for several hours (Baker and Christensen, 1988; Block, 1983; McCoy, 1983). Due to its non-toxic properties, this compound is also used in hair shampoos. An additional advantage of the proposed methodology is that the retention of the antimicrobial compound on the particle surfaces makes it easier to recover and reuse the non-spent chemical, therefore substantially reducing the environmental load in the discharged water.

The goal of the present work was thus to develop and characterize new functionalized microparticles that transport the biocide and to assess their efficacy against planktonic and biofilm microbial cells.

4.2. Material and methods

4.2.1. Reagents

Polyethyleneimine (PEI – molecular weight of 750 000) 50% (w/v) in water, Poly(sodium 4-styrenesulfonate) (PSS - molecular weight of 70 000) and boric acid were obtained from Sigma-Aldrich. BDMDAC (molecular weight of 339.9) was obtained from Fluka. All chemicals were used without further purification. Polystyrene (PS)-core particles 4.37 ± 0.07 μm 10% (w/v) aqueous solution were obtained from Microparticles GmbH.

4.2.2. Particles production process

The particles were prepared using the LbL self-assembly technique. This technique uses electrostatic attraction and complex formation between polyanions and polycations to form supramolecular multilayer assemblies of polyelectrolytes. The first stage of shell fabrication involves step-wise deposition of polyelectrolytes from aqueous solutions. The polyelectrolyte multilayer film is formed by the alternate adsorption of oppositely charged layers on to the particle. After each adsorption step, the non-adsorbed polyelectrolyte in solution is removed by repeated centrifugation or filtration and washing (Donath et al., 1998).

The oppositely charged electrolytes, polyethyleneimine (PEI), sodium polystyrene sulfonate (PSS) and BDMDAC were assembled on PS cores, in a process that comprises 3 steps (Figure 4.1). PS particles were allowed to interact with the PEI solution (1 mg/mL in borate buffer solution) for 20 min, and then washed in 0.1 M borate buffer solution pH 9 to remove the excess polymer. After this procedure, the core positively charged was used for the deposition of the polyanion PSS, followed by the BDMDAC, both solutions at 1 mg/mL in borate buffer pH 9. The adsorption steps were carried out by adding the polymer solution to the PS cores for 20 min, centrifuging at 2880 g for 4 min and resuspending them in borate buffer pH 9. This step was repeated twice.
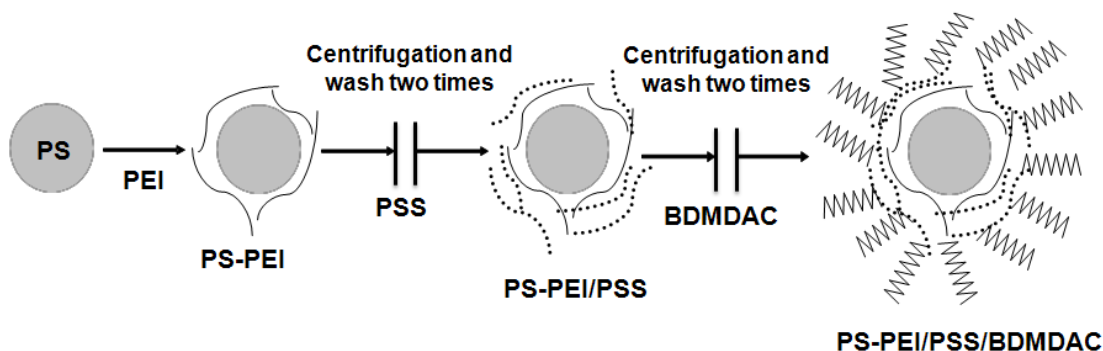
Figure 4.1. Schematic representation of the particles production process. (PEI - polyethyleneimine, PSS - sodium polystyrene sulfonate and BDMDAC).

Borate buffer (0.1 M) at pH 9 was the solvent used in the whole process. It was selected due to the ionic strength of the solution as well as the pH value that allows a better LbL process by promoting the right superficial charge for the different molecules intervening in the process.

4.2.3. Quantification of the amount of biocide in particles

For the quantification of BDMDAC on the surface of coated particles, a high performance liquid chromatography (HPLC) method was developed. The HPLC system consisted of a JASCO PU-2080 plus ternary pump, a manual injector equipped with a 20 μL sample loop and a JASCO MD-2015 plus diode array detector. A Jasco ChromPass Chromatography data system (version 1.8.6.1) allowed to control the equipment and the data processing. The analytical column was CC 250/4 Nucleosil 100-5 C18. The mobile phase consisted of methanol/water pH 1 with ortho-phosphoric acid 85% (v/v). The flow rate was 1.0 mL/min and the detector was set at 210 nm. A calibration curve standards was assessed using different BDMDAC concentrations (50, 70, 100, 200, 250, 300 mg/L) in 0.1 M borate buffer solution pH 9. All the samples were injected in 0.1 M borate buffer solution pH 9. The concentration of BDMDAC adhered to the particles, was assessed as the difference between the BDMDAC solution concentration before (1000 mg/L) and after contact with the particles.

4.2.4. Particles characterization - cryoSEM and X-ray microanalysis

CryoSEM and X-ray microanalysis were performed to analyze the coated particles integrity/morphological characteristics and surface composition, respectively. The samples were analyzed by CryoSEM (Model Gatan ALTO 2500) at Centre for materials characterization from the University of Porto (CEMUP).

4.2.5. Particle characterization - number and volume size distribution

The size distribution of the particles was determined in a Coulter Particle Size Analyzer (model LS 230 – small volume module plus) by Laser Diffraction. The analysis of the particle size was considered in terms of volume and number distribution. The experiments were repeated at least at three different occasions.

4.2.6. Particles characterization - zeta potential.

The zeta potential of the particles was determined using a Nano Zetasizer (Malvern instruments, UK). The zeta potential was measured by applying an electric field across the particles solution. Particles in the aqueous dispersion with non-zero zeta potential migrated toward the electrode of opposite charge with a velocity proportional to the magnitude of the zeta potential. The experiments were repeated at least at three different occasions.

4.2.7. Microorganism

Pseudomonas fluorescens was isolated from a drinking water distribution system and identified by 16 S ribosomal DNA sequence analysis. *Pseudomonas* spp. are known to be good biofilm producers and major microorganisms found in industry (Simões et al., 2005; Simões et al., 2008b). The optimal growth conditions were 27 ± 3 ºC, pH 7, and glucose as the main carbon source. The *P. fluorescens* strain was cryopreserved in a refrigerated chamber at -80 ºC, in a mixture of nutrient broth and 15% (v/v) of glycerol. Bacteria propagation was obtained by removing an inoculum from the cryovial. The

bacteria were then distributed evenly over the surface of Plate Count Agar (PCA) and incubated for 24 h at 27 ± 3 ºC.

4.2.8. Antimicrobial Tests with Planktonic Cells

The bactericidal effect of the BDMDAC coated particles (PS-PEI/PSS/BDMDAC) was tested by comparison with the effect of the non-coated particles. Planktonic cells were obtained from solid medium and resuspended in sterile saline solution (0.85 % NaCl) to an optical density (610 nm) of approximately 0.2 ± 0.02 (bacterial cell density of approximately 1.41×10^8 CFU/mL). The suspension was serially diluted to 10^{-5}. An aliquot of 1.0 mL was collected and used to test the antimicrobial effect of the coated particles. BDMDAC coated particles were tested at different concentrations. Control experiments were performed with bacteria in saline solution and in contact with PS-PEI/PSS particles. The antimicrobial effect of the different systems was evaluated at different incubation times (0, 30 and 60 min). After each incubation time, 100 µL of sample were spread on PCA and incubated for 24 h at 27 ± 3 ºC. The viable cells were counted to assess the antimicrobial effect.

The evaluation of the minimum amount of BDMDAC needed for effective microbial reduction was carried out through the determination of the survival ratio (ratio between CFUs in the antimicrobial test and CFUs in the control; CFU of the microbial population after different periods of exposure to BDMDAC coated particles. The mean and the standard deviation values were determined from the arithmetic values of the survival ratio. The experiments were repeated at three different occasions for every condition tested.

4.2.9. Biofilm set-up - chemostat

Biofilms were developed in a well stirred continuous reactor at 27 ± 3 °C. *P. fluorescens* was grown in a 4 L polymethyl methacrylate (Perspex) fermenter, aerated (air flow rate = 1.8 L/min) and agitated with a magnetic stirrer (P-Selecta Agimatic-S) (Figure 4.2 and Figure 4.3). The fermenter was continuously fed with 0.40 L/h of a sterile nutrient solution consisting of 50 mg/L glucose, 25 mg/L peptone and 12.5 mg/L

yeast extract, in 0.2 M phosphate buffer at pH 7. The bacteria were grown in the fermenter by adding 500 mL of bacterial suspension (optical density 610 nm = 1.0) to 3.5 L of saline solution for approximately 3 h before the beginning of the continuous feeding process. The dilution rate of 0.1 h^{-1} ensured that biofilm activity predominated over that of planktonic cells (Simões et al., 2003). Twelve slides (1.5 cm $\times$ 2 cm and 1 mm thick) of polyvinylchloride (PVC) were vertically placed within the bacterial suspension for 7 days for biofilm growth. The slides were degreased, rinsed twice with water and sterilized before they were suspended in the fermenter using a device that enabled their removal for biofilm sampling. The selection of initial growth conditions and system parameters were based on previous experiments (Simões et al., 2003).
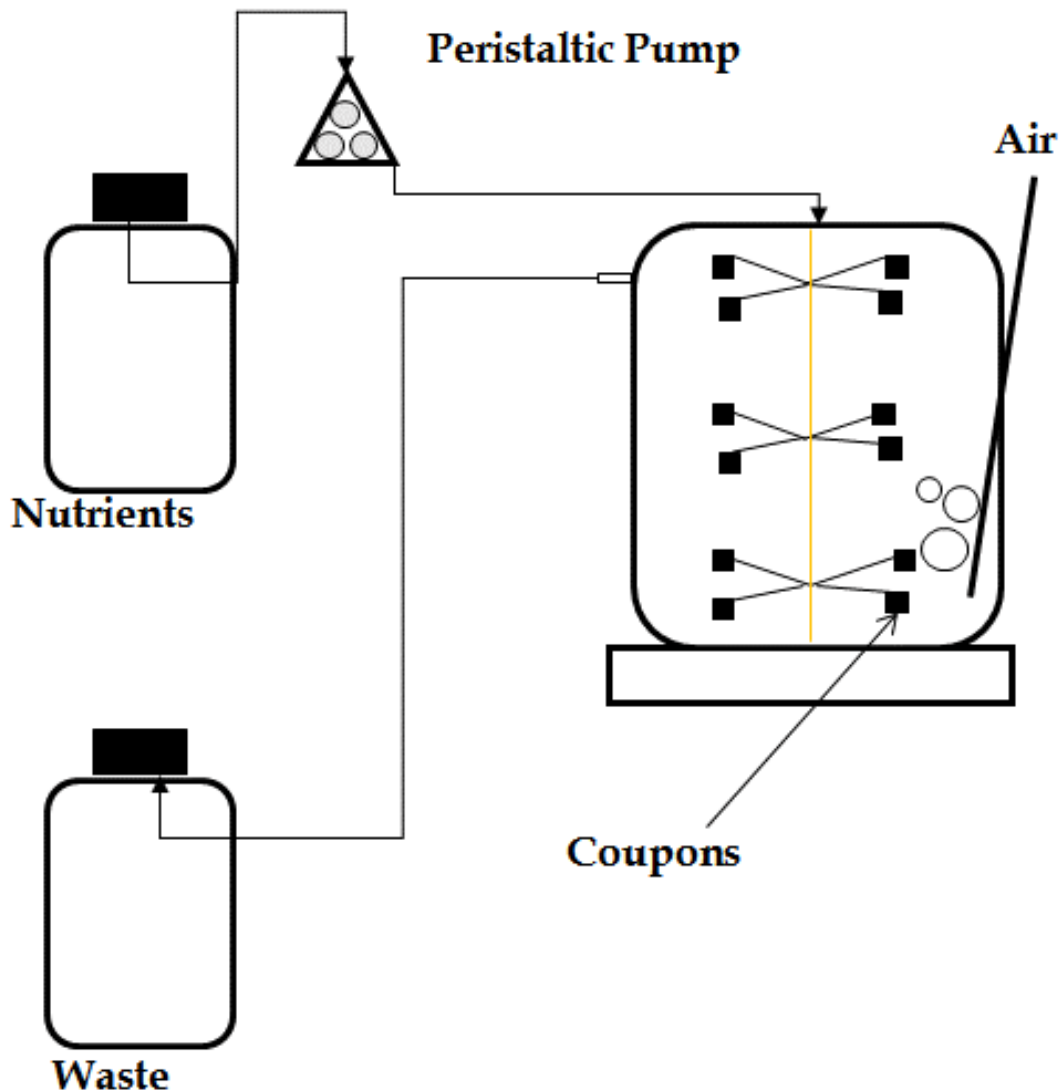
Figure 4.2. Schematic representation of the chemostat.
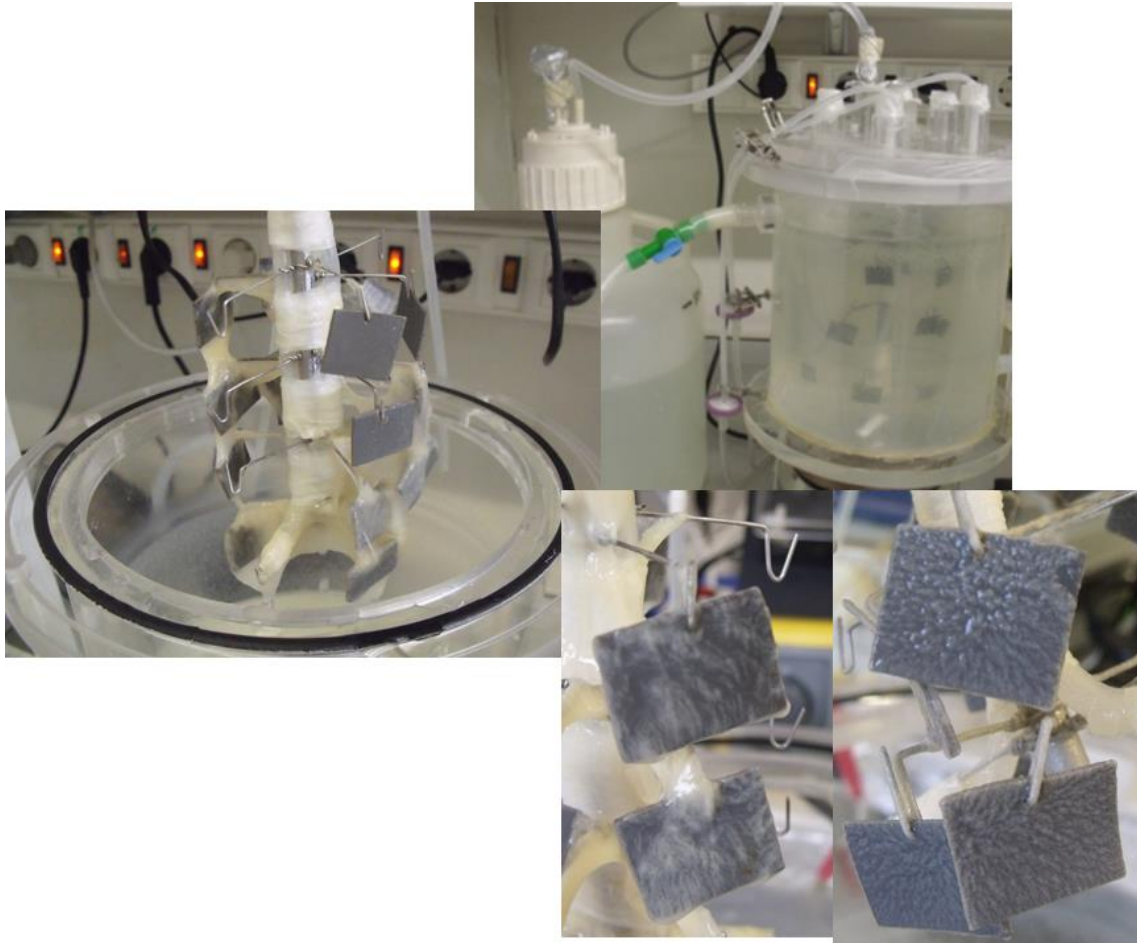
Figure 4.3. Photograph of a chemostat set-up.

4.2.10. Biofilm treatment

After biofilm development, the biofilm-covered slides were carefully transferred to a closed flask that contained the BDMDAC coated particles solution (8×10^7 particles/mL). The flask was placed in an orbital shaker throughout the chemical treatment, to ensure the same temperature and agitation conditions as in the fermenter. Some biofilm-covered slides were placed in saline solution for control assays and in solution of particles at the same concentration but without BDMDAC. The biofilms were exposed to free BDMDAC and to BDMDAC coated particles at different concentrations and independent periods of 30 and 60 min (Figure 4.4). Afterwards, the PVC slides with accumulated biofilm were carefully removed from the solution containing the QAC. Biofilm control action was measured as the variation in the mass deposited during the

treatment period and in the colony forming counts (CFU). The experiments were repeated at three different occasions for every condition tested.
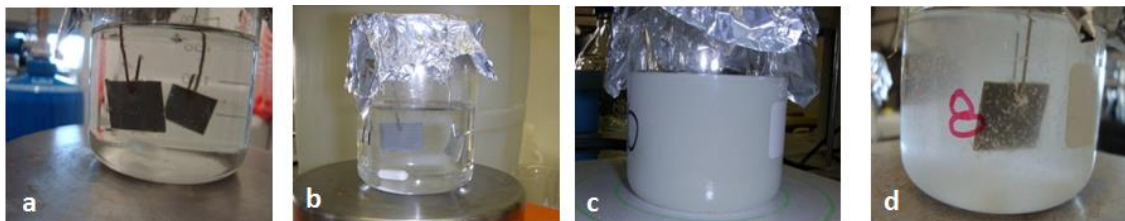
Figure 4.4. Photograph of biofilm treatment, a) control with saline solution, b) free BDMAC, c) microparticles without BDMDAC and d) BMDAC coated microparticles.

4.2.11. Antimicrobial tests with biofilms

In each experiment and immediately after the 30 or 60 min of antimicrobial treatment, two PVC slides were sampled and the biofilms that covered the slides were completely scraped off using a metal device, exposed to an ultrasonic bath at 35 kHz for 15 min (Transsonic 420, Elma) and resuspended in 25 mL of saline solution. The biofilm suspensions were vortexed (IKA TTS2) for 30 s with 100% input and immediately used to assess the biofilm bacteria culturability. The bacterial samples were diluted to the adequate cellular concentration in sterile saline solution. A volume of 100 μL of the bacterial diluted suspension were transferred onto PCA plates. Colony enumeration was carried out after 24 h at 27 ± 3 ºC and the survival ratio was assessed as referred to above for the planktonic tests.

4.2.12. Statistical analysis

The data were analysed using the statistical program SPSS 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Because low samples numbers contributed to uneven variation, nonparametric Wilcoxon test procedure was used to compare the different conditions tested. Statistical calculations were based on confidence level equal or higher than 95 % ($P < 0.05$ was considered statistically significant).

4.3. Results and discussion

This study aims to show the potential of a new antimicrobial strategy to control planktonic cells and biofouling layers based on the principle of drug-delivery systems. The selected antimicrobial chemical is a quaternary ammonium compound (QAC) that is normally used as an industrial cleaner/disinfectant, such as in re-circulating cooling water systems (Bull et al., 1998). BDMDAC has detergent and antimicrobial properties, acting on multiple biochemical targets of the bacterial cells. BDMDAC can disrupt cell membranes, interrupt proteins functions, stimulate the release of intracellular constituents and induce cell autolysis (Ferrer and Furlong, 2001; Ishikawa et al., 2002).

The particles (with and without BDMDAC coating) were characterized in order to assess potential changes induced by the antimicrobial coating process. CryoSEM was used to visualize the morphology of the particles as well as the presence of an external layer (BDMDAC and/or PSS). This technique allows the analysis of hydrated samples conversely to traditional SEM. X-ray microanalysis coupled with CryoSEM was used to confirm the elemental constitution of the particles surface. CryoSEM images of PS-PEI/PSS and PS-PEI/PSS/BDMDAC particles show that they are spherical and have a rough surface both in the presence and absence of BDMDAC (Figure 4.5). X-ray microanalysis indicates that the external layer was mainly composed by carbon and its content is considerably higher in PS-PEI/PSS/BDMDAC particles when compared with PS-PEI/PSS particles. This result indicates that the carbon content on the particles surface increases significantly when coated with BDMDAC. This is an expected phenomenon due to the long carbon chain of BDMDAC (C_{14}). This conclusion is reinforced by the HPLC results and by the zeta potential of the particles. HPLC experiments demonstrated that each particle carried 5.4×10^{-8} mg/L of BDMDAC. The zeta potential of PS-PEI/PSS was -34 ± 4 mV and of PS-PEI/PSS/BDMDAC was -21 ± 5 mV. It was expected that the interaction between BDMDAC and the particles induced a shift in the zeta potential from negative to positive values. However, this shift was not very pronounced. PSS is a polyanion inducing high negative zeta potential values (Tedeschi et al., 2004; Volodkin et al., 2004), while each BDMDAC molecule only provides one positive charge to link to the particle. This fact could be justified by hydrophobic interactions between the carbon chains of BDMDAC and water that blocks the access of more BDMDAC molecules that bind electrostatically to the free negative charges of PSS.
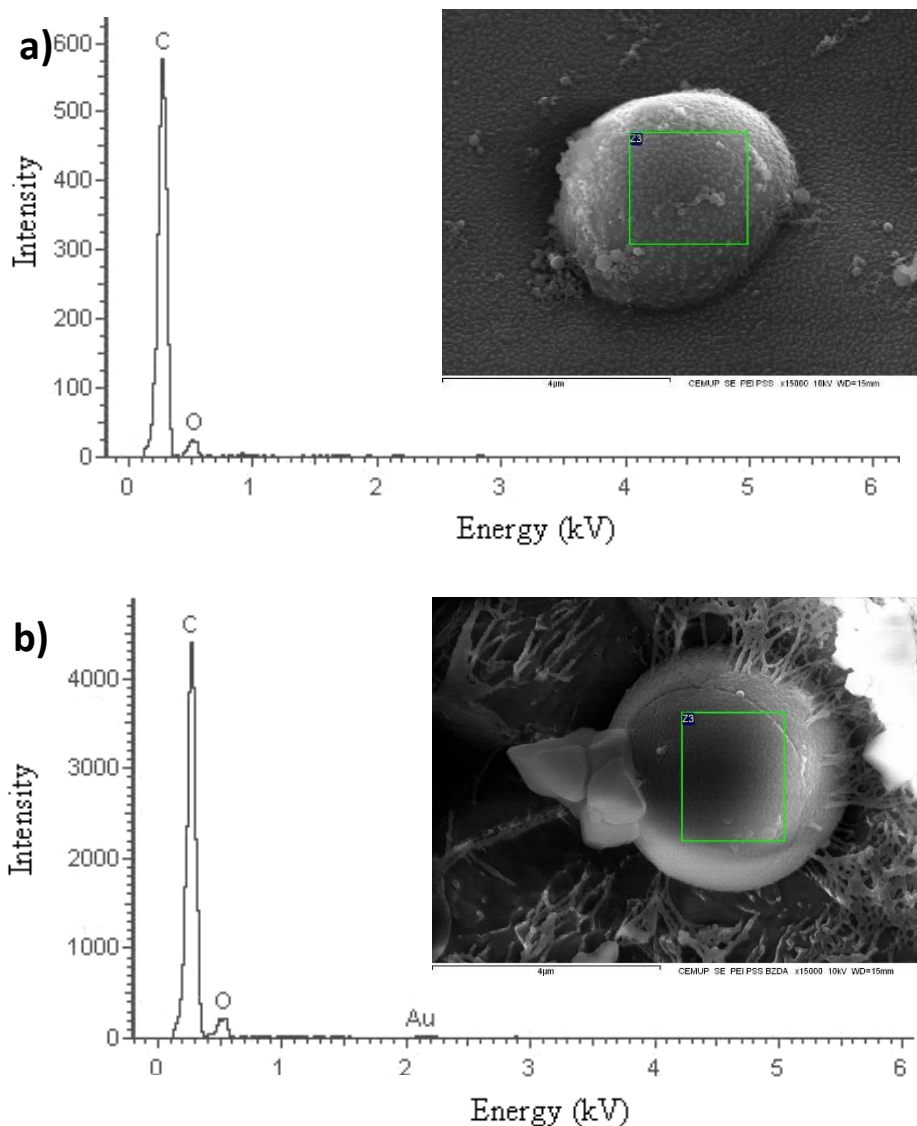
Figure 4.5. CryoSEM images (×15000 magnification; 10 kV) and X-ray microanalysis of PS-PEI/PSS (a) and PS-PEI/PSS/BDMDAC particles (b).

In order to assess the effect of the BDMDAC coating process on the physical characteristics of the particles, the size distribution of BDMDAC coated and uncoated particles was assessed (Figure 4.6). Three particles size distributions could be found for PS-PEI/PSS particles with sizes of 3.0, 4.4 and 15.0 µm. In the case of PS-PEI/PSS/BDMDAC, again three populations were observed, but here two of them are well defined (3.0 and 4.4 µm). A wider distribution could be seen between 6 µm and 20 µm (Figure 4.6). This can be related to the particle aggregation due to hydrophobic interactions between the BDMDAC carbon chains. However, when considering the size distribution in number only the two populations of smaller diameters (3.0 and 4.4 µm)

are significant, indicating that the number of particles with a diameter of 20 μm is not significant ($P > 0.05$), for the tested particles (Figure 4.6).
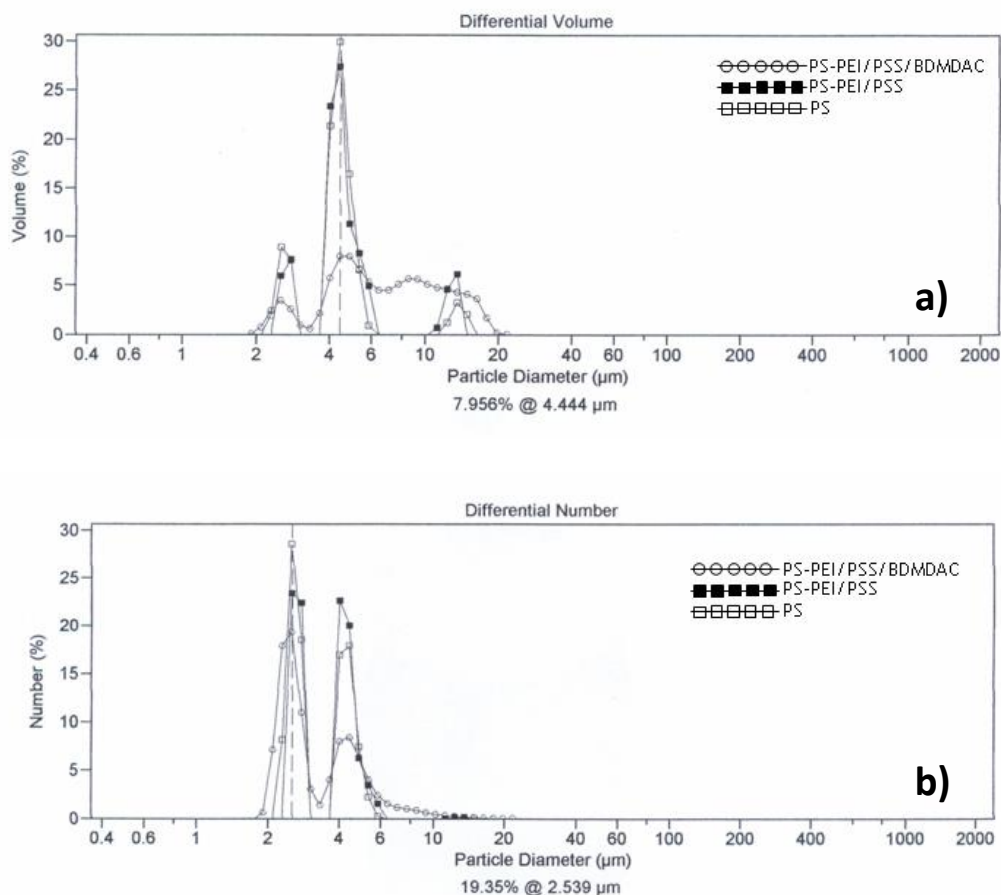
Figure 4.6. Volume distribution (a) of the particles (PS, PS-PEI/PSS and PS-PEI/PSS/BDMDAC); Size distribution (b) of the particles in number (PS, PS-PEI/PSS and PS-PEI/PSS/BDMDAC).

The effect of the QAC was first investigated on planktonic cells. The effect was determined as a survival ratio between the CFU for the antimicrobial test and the CFU in the saline solution (Figure 4.7). The results obtained for the mixture of PS-PEI/PSS particles with the suspension of the *P. fluorescens* showed that they have no bactericidal effect (survival ratio around 1). The number of bacteria in the presence of these particles was approximately the same as for the saline solution ($P > 0.05$). Also, the concentration of viable cells was invariable over time (1.10×10^3 CFU/mL). The particles coated with BDMDAC (PS-PEI/PSS/BDMDAC) showed a clear antimicrobial effect (survival ratio = 0). The minimum bactericidal concentration (MBC) for a 30 min contact time was 9.2

mg/L, while the MBC for a 60 min contact time was 6.5 mg/L. The planktonic tests indicate a pronounced antimicrobial effect of BDMDAC when associated with the particles. BDMDAC has a strong antimicrobial activity comparatively to other QAC`s (Ishikawa et al., 2002; Méchin et al., 1999; Simões et al., 2006).
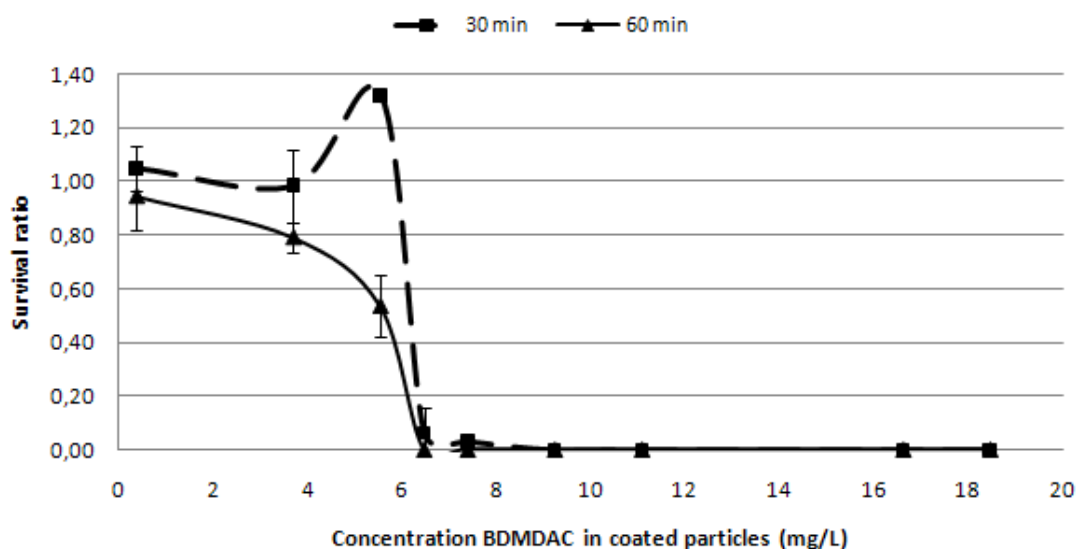
Figure 4.7. Survival ratio of planktonic *P. fluorescens* exposed to different concentrations of the BDMDAC adsorbed in particles for two different exposure periods. Each symbol indicates the mean ± the standard deviation value of three independent experiments.

The effects of the application of free BDMDAC and BDMDAC coated particles at different concentrations, for 30 and 60 min, against biofilms formed on PVC slides were assessed by determining the survival ratio of the biofilm population (Figure 4.8). The biofilm exposure to the biocidal particles with a very low concentration of BDMDAC (0.87 mg/L) resulted in a viability decrease of 60.5% and 66.5% of the total biofilm population for 30 and 60 min exposure time, respectively. Comparing with the application of equivalent concentration and exposure periods of free QAC, it was verified that the free BDMDAC had a moderately higher antimicrobial activity than the BDMDAC coated particles (viability decrease of 63.4% and 70.3% for a 30 min and 60 min exposure period, respectively). However, the differences observed were not statistically significant ($P > 0.05$). In a previous report (Simões et al., 2005), it was

verified that the exposure of *P. fluorescens* flow-generated biofilms for 30 min to the QAC cetyl trimethyl ammonium bromide at 328 mg/L, only promoted the inactivation of 65% of the biofilm activity. Moore et al. (2008) applied QAC`s at concentrations higher than 100 mg/L to control domestic drain biofilms; however, the prolonged-sublethal exposure to the chemicals resulted in susceptibility decreases.
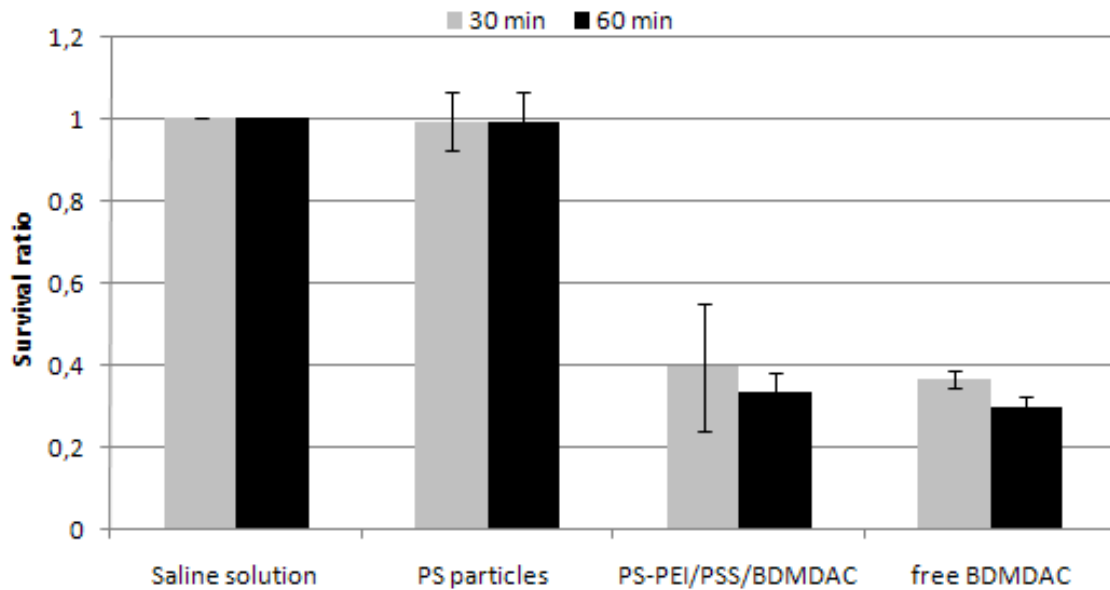
Figure 4.8. Survival ratio of *P. fluorescens* biofilms exposed to control conditions (saline solution and PS particles) and to 0.867 mg/L of BDMDAC coated particles and free BDMDAC for two different exposure periods. Each symbol indicates the mean ± the standard deviation value of three independent experiments.

Additional experiments were performed in order to assess the effect of increasing biocide concentration on biofilm control. With this purpose, two additional concentrations of BDMDAC, free and in coated particles (6.33 and 11.7 mg/L), were applied for 60 min to biofilms (Figure 4.9). The BDMDAC coated particles decreased the number of biofilm viable cells in 80.6% (6.33 mg/L) and 87.2% (11.7 mg/L) while the free QAC decreased the viable cells in 85.6% (6.33 mg/L) and 100% (11.7 mg/L). The differences in the decrease of cell viability by the application of free BDMDAC or BDMDAC coated particles were not statistically significant ($P > 0.05$). The moderately

higher antimicrobial effect of the free QAC could be related with diffusion limitations associated with the particle size. There are evidences that the particle size and shape can influence antibacterial activity (Fatin-Rouge et al., 2004; Pal et al., 2007; Sanders et al., 2000). It is conceivable that reducing the particle size or increasing the antimicrobial concentration to the levels applied in industrial systems (Baker and Christensen, 1988; Block, 1983; McCoy, 1983) will increase the antimicrobial effect.
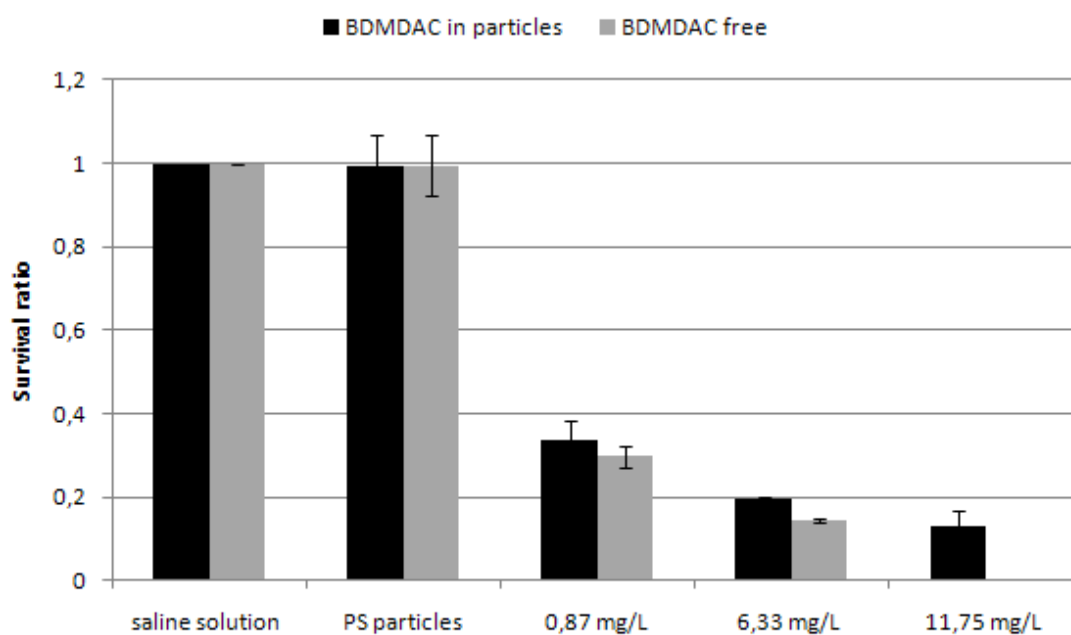
Figure 4.9. Survival ratio of *P. fluorescens* biofilms exposed to control conditions (saline solution and PS particles) and to BDMDAC coated particles (PS-PEI/PSS/BDMDAC) and free BDMDAC at three different concentrations, for a 60 min exposure period. Each symbol indicates the mean ± the standard deviation value of three independent experiments.

The environmental aspects of the current use of antimicrobials are of severe concern because of their residual presence in surface and ground waters and the consequent propagation to the food-chain, with risks to the public health. The controlled application and reuse of antimicrobials based on highly efficient strategies might avoid the dissemination of antimicrobial resistance. The prolonged exposure of microorganisms to sub-lethal concentrations promotes antimicrobial resistance and

cross-resistance events (McDonnell and Russell, 1999; White and McDermott, 2001). There are several reports indicating the ability of bacteria to acquire resistance to QAC`s (Ishikawa et al., 2002; Méchin et al., 1999). Many studies have demonstrated that bacteria are capable of adapting to disinfectants used in industrial settings after prolonged exposure to sublethal concentrations (Aase et al., 2000; To et al., 2002). Loughlin et al. (2002) reported the ability of *P. aeruginosa* to adapt to increasing concentrations of benzalkonium chloride and verified the co-resistance to other membrane-active biocides. Also, the economical costs associated with the continuous application of antimicrobial chemicals should be considered. The strategy used in this study to control planktonic and biofilm cells will allow the rational use and reuse of antimicrobials. After 18 months in borate buffer pH 9 (kept at 4 ºC), the particles coated with BDMDAC released only 15% of the QAC. The results obtained in the present study clearly demonstrate that this novel biofilm control strategy may have potential public health, environmental and economical benefits by effectively limiting the levels of biocides used in cleaning and disinfection practices.

Possible difficulties in the application of this antimicrobial strategy may come from the current particles costs (368 € per 15 mL of particle solution). Cheaper particle cores, such as clays and magnetite are attractive for biocide immobilization. The first ones are quite inexpensive, while the latter present the advantage of being more easily recovered from the water by magnetic separation. Such improvements will favour the large scale application of this process against both planktonic and biofilm cells.

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Chapter 5

Biofilm control in a chemostat using calcium carbonate microparticles with immobilized biocide

Objective: The aim of the present work was to develop new functionalized calcium carbonate microparticles coated with biocide, and assess their efficacy against biofilms of *P. fluorescens* formed in a well stirred reactor.

5.1. Introduction

In previous study (chapter 4) PS microparticles were used as a model core. These particles are expensive. Therefore, calcium carbonate microparticles ($CaCO_3$) (diameter: 3 µm), functionalized by the same LbL assembly technique were developed and tested. These microparticles have the advantage of being cheaper, porous and made of a highly abundant mineral in nature. Calcium carbonate is subject of many studies due to its importance in various fields as medicine, food industry, plastic industry, etc. (Mitsutaka, 2001). Calcium carbonate has three kinds of anhydrous crystalline polymorphs: vaterite (polycrystalline spheres), aragonite (needles), and calcite, with rhombohedral, orthorhombic and hexagonal structures (Helmut, 2003; Volodkin et al., 2004a; Volodkin et al., 2004b). The result of the crystallization process was found to be strongly dependent on the experimental conditions, such as: the type of the salts used, their concentration, pH, temperature, rate of mixing the solutions, and the intensity of agitation of the reaction mixture, parameters affecting the rate of the nucleation process (Sukhorukov et al., 2004; Volodkin et al., 2004a). Alcohols like ethanol, isopropanol or diethylene glycol were also found to influence the $CaCO_3$ morphology through $CaCO_3$ polymorph selection, by preventing the transformation of complex shaped vaterite aggregates into calcite.

Following previous results obtained with polystyrene microparticles, the aim of the present work was to develop new functionalized calcium carbonate microparticles nanocoated with a biocide layer, and assess their efficacy against biofilm microbial cells. The LbL assembly technique was used to produce these microparticles and the chosen biocide was BDMDAC, a surface-active agent and low-cytotoxic disinfectant that is often used in industrial applications (concentrations around 50 mg/L).

5.2. Material and methods

5.2.1. Reagents

Reagents were the same as described in section 4.2.1. Calcium carbonate microparticles ($CaCO_3$) with a diameter of 2-4 µm were obtained from PlasmaChem GmbH. All chemicals were used without further purification.

5.2.2. Particles manufacture process

Both particles were prepared using the LbL self-assembly technique as describe in section 4.2.2. The oppositely charged electrolytes, PEI, PSS and BDMDAC, were assembled on both calcium carbonate and PS cores, in a process that comprises 3 steps.

5.2.3. Quantification of the amount of biocide in particles

To quantify BDMDAC on the surface of coated particles, a high performance liquid chromatography (HPLC) method was assembled as described in 4.2.3.

5.2.4. CryoSEM analysis of particles

CryoSEM was performed to analyze the coated particles integrity and morphological characteristics. CryoSEM analyses were performed in a Gatan ALTO 2500 Model, at the Center for Materials Characterization from the University of Porto (CEMUP).

5.2.5. Zeta potential of particles

The zeta potential of the particles was determined using a Nano Zetasizer (Malvern instruments, UK) as described in section 4.2.6.

5.2.6. Microorganism

The *Pseudomonas fluorescens* strain chosen for this work was the same as described in 4.2.7.

5.2.7. Biofilm set-up

Biofilms were developed in a well stirred continuous reactor at 27 ± 3 °C. *P. fluorescens* was grown in a 4 L polymethyl methacrylate (Perspex) fermenter, suitably aerated and magnetically agitated as descrive in section 4.2.9.

5.2.8. Antimicrobial test with biofilm

After biofilm development, the biofilm-covered slides were carefully transferred to a closed flask that contained the BDMDAC coated particles solution (8×10^7 particles/mL). The flask was placed in an orbital shaker throughout the chemical treatment, to ensure the same temperature and agitation conditions as in the fermenter. Some biofilm-covered slides were placed in saline solution for control assays and in a solution of particles at the same concentration but without BDMDAC. For comparison, additional experiments were performed with free BDMDAC at the same concentration as in the coated particles. The biofilms were exposed to BDMDAC coated particles at different concentrations (8.33 mg/L and 11.75 mg/L) and at independent periods of 60 min. Afterwards, the PVC slides with accumulated biofilm were carefully removed from the solution of BDMDAC-coated particles. Biofilm control action was measured as the variation in the colony forming counts (CFU).

5.2.9. Biofilms analysis

In each experiment and immediately after 60 min of BDMDAC-coated particles treatment, two PVC slides were sampled and the biofilms that covered the slides were completely scraped off using a metal device, exposed to an ultrasonic bath at 35 kHz for 15 min (Transsonic 420, Elma) and resuspended in 25 mL of saline solution. The biofilm suspensions were vortexed (IKA TTS2) for 30 s with 100% input and immediately used to assess the number of CFU. The bacterial samples were diluted to the adequate cellular concentration in sterile saline solution. A volume of 100 μL of the bacterial diluted suspension were transferred onto PCA plates. Colony enumeration was carried out after 24 h at 27 ± 3 ºC. The evaluation of the microbial reduction was carried out through the determination of the survival ratio (ratio between CFUs in the antimicrobial test and CFUs in the control; CFU- colony formation units) of the microbial population after different periods of exposure to BDMDAC coated particles.

5.2.10. Statistical analysis

The data were analysed using the statistical program SPSS 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Because low samples numbers contributed to uneven variation, nonparametric Wilcoxon test procedure was used to compare the different conditions tested. Statistical calculations were based on confidence level equal or higher than 95 % ($P < 0.05$ was considered statistically significant).

5.3. Results and discussion

With the aim of making industrial cleaning/disinfectant methods more efficient, $CaCO_3$ microparticles coated with BDMDAC were produced, a quaternary ammonium compound frequently used as an industrial cleaner/disinfectant. PS microparticles were used form comparison. *P. fluorescens* was used in this study as a model bacterium not only because it is a well studied, Gram-negative bacterium ubiquitous in nature, medical and industrial environments, but also because it has a strong ability to form disinfectant-resistant biofilms (Simões et al., 2008). Thus, *P. fluorescens* has a strong potential to cause serious problems in a wide range of areas in its planktonic and biofilm states (Hsueh et al., 1998; Simões et al., 2010; Tuttlebee et al., 2002).

The characterization of the particles surface with and without BDMDAC coating was done by CryoSEM (Figure 5.1). This technique allows the analysis of hydrated samples, with the advantage, of the freezing process being so fast, that the samples structures preserve their integrity.

CryoSEM images of PS and PS-PEI/PSS/BDMDAC particles show that they are spherical and have a rough surface in both the presence and absence of BDMDAC. CryoSEM images of $CaCO_3$ particles and $CaCO_3$- PEI/PSS/BDMDAC particles show that these particles are spongier than the PS ones (Figure 5.2).

(a)   (b)
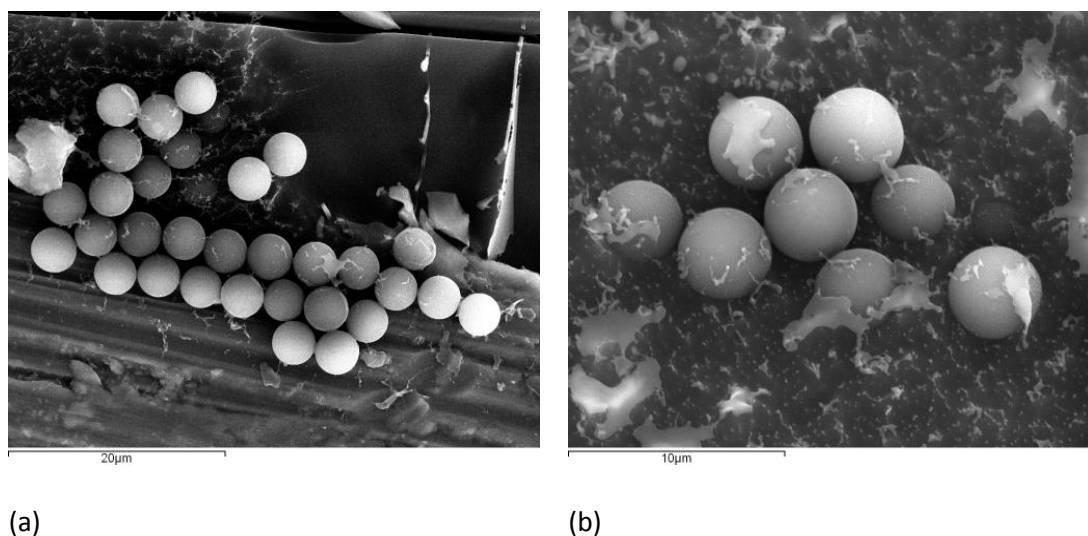
Figure 5.1. CryoSEM images (×2500 magnification; 15 kV) of PS particles (a) and PS-PEI/PSS/BDMDAC particles (×5000 magnification; 15 kV).

(a)   (b)
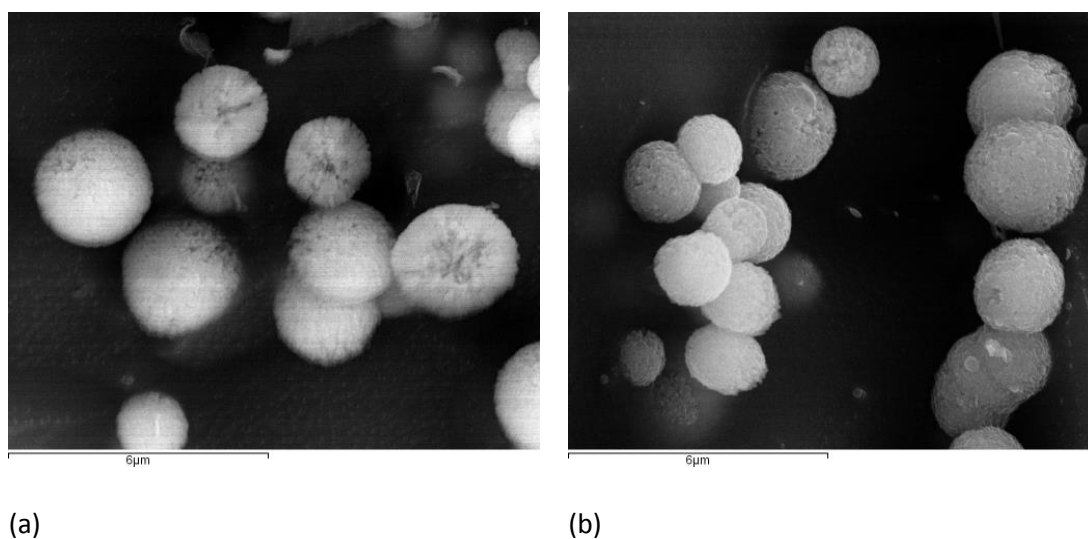
Figure 5.2. CryoSEM images (×10000 magnification; 15 kV) of $CaCO_3$ particles (a) and $CaCO_3$-PEI/PSS/BDMDAC particles (b).

Higher amplifications of CryoSEM images of $CaCO_3$ particles with and without biocide allow the observation that the particles surfaces with biocide are smoother than the surfaces of particles without biocide (Figure 5.3)**.** In fact, this is an expected phenomenon since we are adding layers that stay strongly attached to the particles surfaces and will cover up those referred surfaces. Consequently, the biocide is bounded

to the microparticles by ionic interactions, using the LbL technique, and therefore it will not be released from the particles when interacting with the biological structures.
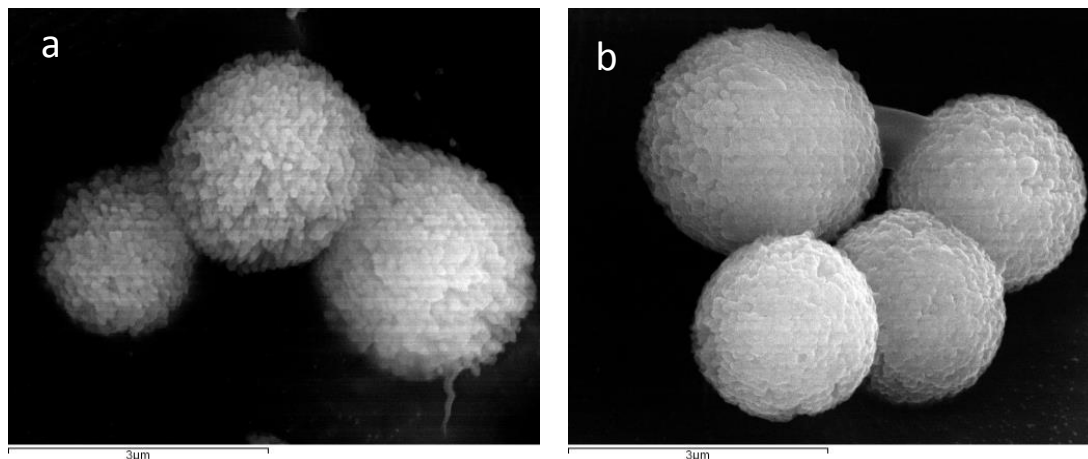
Figure 5.3. CryoSEM images ($\times 20000$ magnification; 15 kV) of $CaCO_3$ particles (a) and $CaCO_3$-PEI/PSS/BDMDAC particles.

This conclusion is reinforced by the HPLC results and by the zeta potential of the particles. HPLC experiments demonstrated that each particle of PS carried 5.4×10^{-8} mg/L of BDMDAC and each particle of $CaCO_3$ carried 1.3×10^{-7} mg/L of BDMDAC. This is expected, since the surface area of $CaCO_3$ is larger.

The zeta potential of PS-PEI/PSS was -34 ± 4 mV and of PS-PEI/PSS/BDMDAC was -21 ± 5 mV. For the particles $CaCO_3$-PEI/PSS and $CaCO_3$-PEI/PSS/BDMDAC the values of zeta were -46 ± 4 mV and -18 ± 3 mV, respectively. Once again, due to their large surface, it is possible to incorporate more PSS in the $CaCO_3$ particles, which is a polyanion capable of inducing high negative zeta potential values (Tedeschi et al., 2004; Volodkin et al., 2004b). It was expected that the particles would shift to positive values of their zeta potential when coated with BDMDAC (a positively charged QAC). However, this did not happen, probably due to the hydrophobic interactions between the carbon chains of BDMDAC and water. These will block the access of more BDMDAC molecules that would bind electrostatically to the free negative charges of PSS. Still, as expected, this shift was more pronounced in $CaCO_3$ particles. This suggests that the antimicrobial action of coated microparticles is promoted by the long carbon chains that are turned out on the particles and the biocide can act on the cell membrane. This was evidenced in chapter 3 when using BDMDAC in solution.

The effects of the application of BDMDAC at different concentrations against biofilms formed on PVC coupons were assessed by determining the biofilm population survival ratio between the CFUs from the biocides and the CFUs from the assays with saline solution (control). The assessment of culturability to characterize the antimicrobial activity of a biocide was already used in the previous chapter.

Biofilms were exposed to two different concentrations of BDMDAC (free and coated on PS particles): 6.33 mg/L and 11.7 mg/L (Figure 5.4).
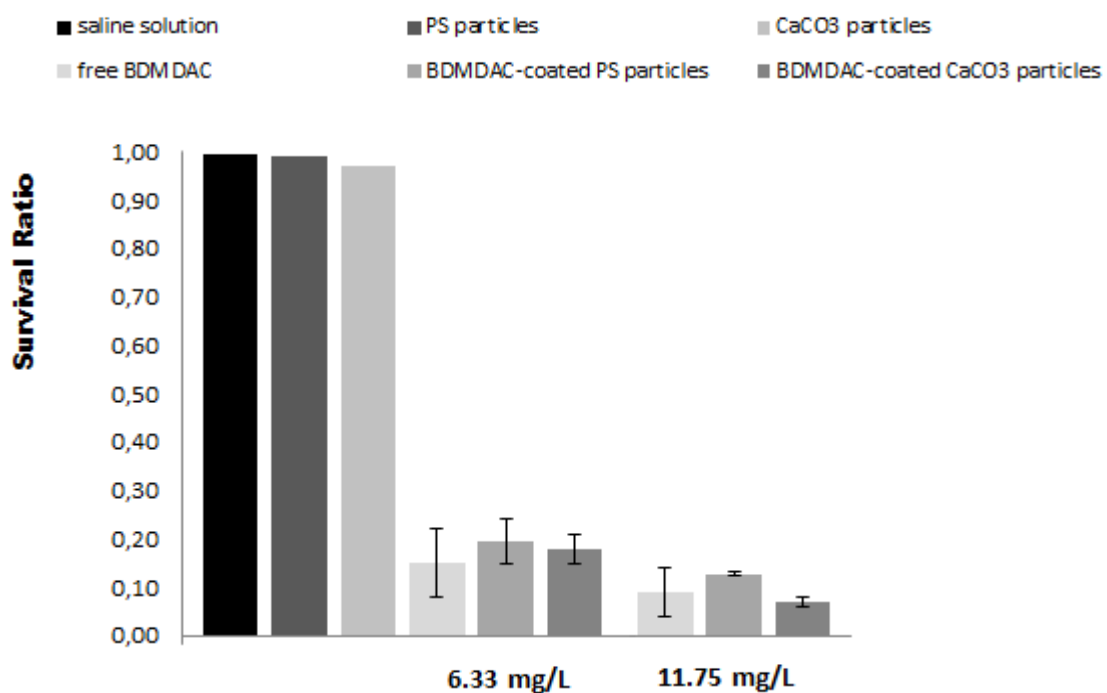
Figure 5.4. Survival ratio of *P. fluorescens* after 1 hour of exposure to free biocide, PS particles coated with BDMDAC and CaCO$_3$ particles coated with the same biocide.

These treatments decreased the number of biofilm viable cells in 80% (6.33 mg/L) and 87.2% (11.7 mg/L).

The same tests were performed for the application of CaCO$_3$ particles coated with BDMDAC. Preliminary results are promising comparatively to the PS microparticles carrying the antimicrobial agent, with the same amount of biocide promoting higher percentage of biofilm inactivation: 81.9% for 6.33 mg/L and 93.3% for 11.75 mg/L of BDMDAC. The particles acted on biofilm control without apparent destruction or biodegradation.

The effects of the application of free BDMDAC had a moderately higher effect on number of viable cells 84.9% (6.33 mg/L) and 91.3% (11.75 mg/L). However, the differences observed were not statistically significant ($P > 0.05$).

In a previous report (Simões et al., 2005), it was verified that the exposure of *P. fluorescens* biofilms during 30 min to the QAC cetyl trimethyl ammonium bromide at 328 mg/L, only promoted the inactivation of 65% of the biofilm activity. Moore and his co-workers (2008) applied QAC`s at concentrations higher than 100 mg/L to control domestic drain biofilms.

Overall, the results obtained in the present study clearly demonstrate that this novel biofilm control strategy may have potential public health, environmental and economical benefits by effectively limiting the levels of biocides used in cleaning and disinfection practices.

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Chapter 6

Application of calcium carbonate biocidal microparticles against biofilms formed in a flow channel

Objective: This study was aimed at producing $CaCO_3$ microparticles with defined physical characteristics and, after BDMDAC immobilization, using those microparticles for the control of *P. fluorescens* biofilms. In this chapter, the biofilms were formed under flowing conditions in a flow cell reactor that mimics the hydrodynamic conditions found in industrial pipes.

6.1. Introduction

Biofilms are unwanted in many situations, particularly in the biomedical, environmental, and industrial fields (Shannon et al., 2008). From the economic and environmental points of view, three of the most important examples of industrial biofouling occur in membrane systems, cooling water tubes and heat exchanger channels (Ferreira et al., 2010; Melo and Flemming, 2010). In industry, biofouling formation causes increased pressure drop and reduced heat transfer efficiency. These problems lead to an increase in the production and maintenance costs. The control and destruction of undesirable biofilms often includes the use of chemical products with antimicrobial properties like biocides and surfactants. However, these substances can be considered harmful for the environment and consequently should be used in as small quantities as possible (Li et al., 2008; Shannon et al., 2008). This study presents recent advances in a new technological approach to minimize the use of antimicrobial agents and their deleterious effects, based on the principle of drug carriers where the antimicrobial chemicals are transported on microparticles.

In chapter 5 $CaCO_3$ microparticles were used to immobilize BDMDAC. These microparticles have the advantage of being cheap, porous and highly abundant in nature. In this study a simple and reproducible procedure was standardized for the preparation of uniform, homogeneous size and nonaggregated microparticles based on the studies of (Sukhorukov et al., 2004; Volodkin et al., 2004a)

The aim of the present work was to test calcium carbonate particles produced *in situ* and functionalized with biocide, and to assess their efficacy against flow-generated biofilms. The LbL assembly was used to coat these microparticles with BDMDAC. Those microparticles were used to control biofilms of *P. fluorescens* formed in a flow cell reactor.

6.2. Material and methods

6.2.1. Reagents

The same that was describe in section 4.2.1. Calcium chloride and sodium carbonate was purchased from Scharlau. All chemicals were used without further purification.

6.2.2. Preparation of CaCO₃ microparticles

Uniform, nearly spherical microparticles of $CaCO_3$ with narrow size distribution were prepared by colloidal crystallization from supersaturated (relative to $CaCO_3$) solution (Sukhorukov et al., 2004; Volodkin et al., 2004a). The process was initiated by rapid mixing of equal volumes of $CaCl_2$ and Na_2CO_3 solutions (0.33 M) at room temperature (Figure 6.1). After intense agitation on a magnetic stirrer, the precipitate was centrifuged at 3202 g for 10 minutes and washed with ultrapure water. A final washing step was performed using 99% ethanol. At least, the particles were dried in an incubator at 60 ºC during 1 h and stored at room temperature.
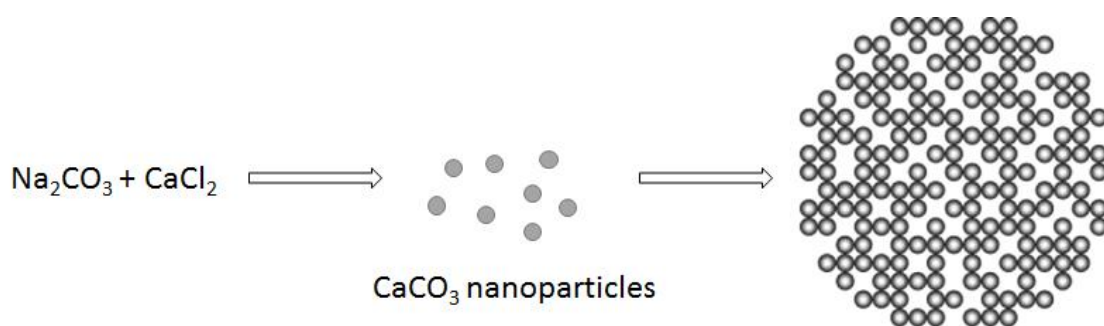
Figure 6.1. Schematic representation of the $CaCO_3$ microparticles manufacture. Based in Volodkin et al. (2004a).

6.2.3 Characterization of CaCO₃ microparticles

6.2.3.1. Mineralogical composition

The mineralogical composition measure with X-ray Diffraction, of the $CaCO_3$ microparticles prepared in this study was provide by LNEG (Laboratório Nacional de Energia e Geologia).

6.2.3.2. Particle manufacture process

Particles were prepared using the LbL self-assembly technique (Caruso, 2001; Decher, 1997; Decher et al., 1992) as described in section 4.2.2.

6.2.3.3. SEM analysis of particles

SEM was performed to analyze the particles integrity and morphological characteristics. SEM analyses were performed using FEI Quanta 400FEG ESEM/EDAX Genesis X4M at the Center for Materials Characterization from the University of Porto (CEMUP).

6.2.3.4. Zeta potential of microparticles

The zeta potential of the particles was determined using a Nano Zetasizer (Malvern instruments, UK). The zeta potential was measured by applying an electric field across the particles solution.

6.2.3.5. Size distribution in number

The size distribution of the particles was determined in a Coulter Particle Size Analyzer (model LS 230 – small volume module plus) by Laser Diffraction. The analysis of the particle size was considered as volume distribution and number distribution. A sample placed in the fluid module is circulated through a sample cell at a constant speed. A beam of laser light shone through the cell is diffracted by particles within the sample, and the forward scattered (or diffracted) light is collected by series of detectors. The distribution of light falling on the sensors enables the size distribution of the sample to be calculated.

### *6.2.4. Quantification of the amount of biocide in particles*

To quantify BDMDAC on the surface of coated particles, a high performance liquid chromatography (HPLC) method was assembled as described in 4.2.3.

6.2.5. Microorganism

P. fluorescens strain chosen for this work was the same as describes in 4.2.7.

6.2.6. Biofilm set-up – flow cell

A pure culture of *P. fluorescens* was grown in a 500 mL shaking flask with 200 mL of growth medium, for 24 h, under optimal growth conditions (T = 27 °C, pH = 7 and glucose as carbon source). After the incubation period, the suspension was diluted in 0.5 L reactor operating continuously. In order to obtain the desired dilution rate to promote biofilm formation the bacterial suspension was diluted using a 4 L Perspex reactor containing distilled and sterilized saline solution (0.85% NaCl). This reactor was continuously inoculated with bacteria coming from the 0.5 L reactor at 10 mL/h using a peristaltic pump. The reactor was continuously fed with 0.40 L/h of a sterile nutrient solution consisting of 50 mg/L glucose, 25 mg/L peptone and 12.5 mg/L yeast extract, in 0.2 M phosphate buffer at pH 7. The dilution rate of 0.1 h^{-1} ensured that biofilm activity predominated over that of planktonic cells (Simões et al., 2003). Twelve coupons (1 cm × 2 cm and 1 mm thick) of polyvinylchloride (PVC) were vertically placed in a flow cell reactor. The diluted bacterial suspension from the 4 L reactor was allowed to recirculate in the flow cell reactor (flow rate was maintained at 420 L/h) in order to form biofilms on the PVC coupons (Figure 6.2 and Figure 6.3).
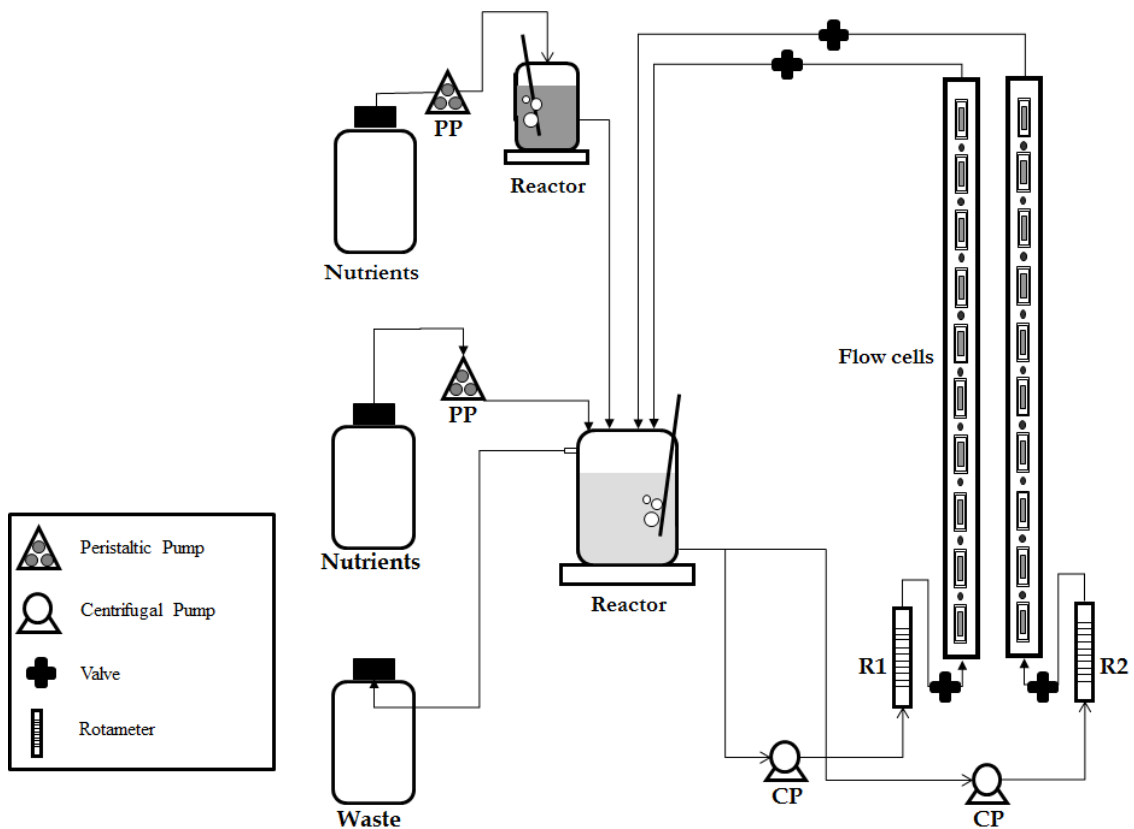
Figure 6.2. Schematic representation of the flow cell reactor system.

Figure 6.3. Photograph of the flow cell set-up.

6.2.7. Antimicrobial tests with biofilms

After biofilm development (14 days), the flow cell was careful emptied and the disinfection of the flow cell was made by recirculation, in closed system of a solution of the BDMDAC free and immobilized in particles at a concentration of 50 mg/L (flow was 0.8 L/h). Control experiments with saline solution were also performed. At 0, 60, 90 and 180 minutes two coupons were taken out of the flow cell.

6.2.8. Biofilm analysis

In each experiment PVC coupons were sampled and their thickness were immediately assessed. Afterwards the biofilms that covered the coupons were completely scraped off using a metal scalpel, exposed to an ultrasonic bath at 35 kHz for 15 min (Transsonic 420, Elma) and resuspended in 10 mL of saline solution. The biofilm suspensions were vortexed (IKA TTS2) for 30 s with 100% input. The homogenised biofilm suspensions were used to determine bacterial culturability and viability.

6.2.8.1. Biofilm thickness

Biofilm thickness was determined using a digital micrometer (VS-30H Mitsubishi Kasei corporation). In this process, a needle connected to the digital micrometer was applied on the top of the biofilm to cross the sample into the bottom of the biofilm. The thickness was obtained through the difference between the readings made on both top and bottom faces of the sample.

6.2.8.2. Culturability assessment

The bacterial samples were diluted to the adequate cellular concentration in sterile saline solution. A volume of 100 μL of the bacterial diluted suspension were transferred onto PCA plates. Colony enumeration was carried out after 24 h at 27 ± 3 ℃.

6.2.8.3. Viability assessment

The Live/Dead® *Bac*Light™ kit (Molecular Probes, L-7012, Leiden, Netherlands) assesses membrane integrity by selective stain exclusion (Simões et al., 2005). After treatment, 300 μL of each diluted suspension were filtered 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 – PI). The dyes were left to react for 7 min in the dark, at 27 ± 1 ℃. The membrane was then mounted on *Bac*Light mounting oil, as described in the instructions provided by the manufacturer. Solutions containing the dyes were previously prepared by dissolving 37 μL of each component in 10 mL of sterile-filtered (pore size 0.22 μm) water. The microscope use for observation of stained bacteria was

a LEICA DMLB2 with a mercury lamp HBO/100W/3 incorporating a CCD camera to acquire images using IM50 software (LEICA) and a $100 \times$ oil immersion fluorescence objective. The optical filter combination for optimal viewing of stained mounts consisted of a 480 to 500 nm excitation filter in combination with a 485 nm emission filter (Chroma 61000-V2 DAPI/FITC/TRITC). A program path (Sigma Scan Pro 5) involving object measurement and data output was used to obtain the total number of cells (both stains) and the number of PI stained cells. Both the total number of cells and the number of PI stained cells on each membrane was estimated from counts of a minimum of 20 fields of view. The range of total cells per field was between 50-200 cells.

6.2.9. Statistical analysis

The data were analysed using the statistical program SPSS 14.0 (Statistical Package for the Social Sciences). The mean and standard deviation within samples were calculated for all cases. Additionally, nonparametric Wilcoxon test procedure was used to compare the different conditions tested. Statistical calculations were based on confidence level equal or higher than 95 % ($P < 0.05$ was considered statistically significant).

6.3. Results and discussion

CaCO$_3$ microparticles coated with BDMDAC were produced for this study. The selection of the calcium carbonate as core was based on its well characterized chemistry, nontoxic nature, low price and easy preparation (Helmut, 2003; Volodkin et al., 2010). A simple and reproducible procedure for preparation of spherical and nonaggreated and highly porous microparticles of CaCO$_3$ was developed based on Sukhorukov et al. (2004) and Volodkin et al. (2004a). This procedure resulted in highly homogeneous spherical CaCO$_3$ microparticles (Figure 6.4).

(a)   (b)
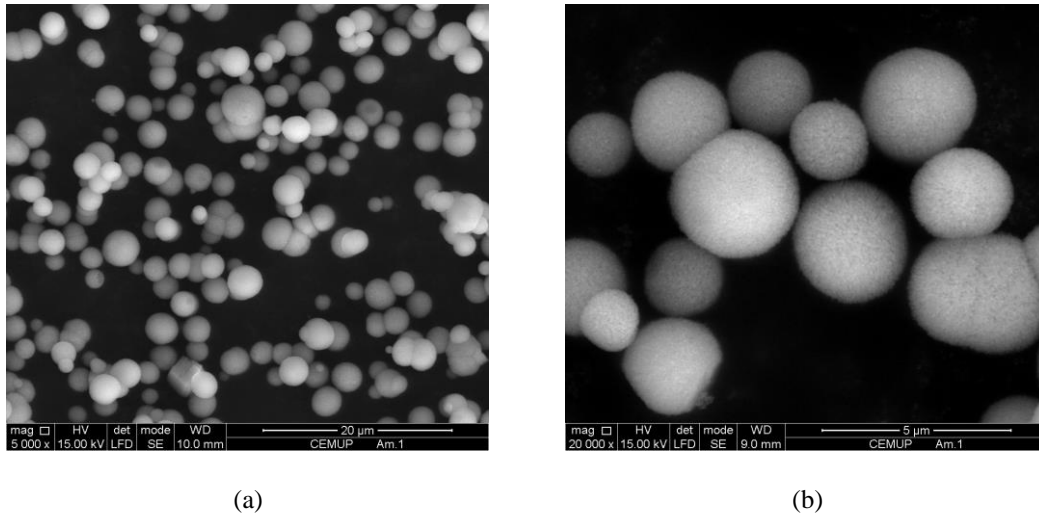
Figure 6.4. SEM images of the CaCO$_3$ produce for this study. (a) ×5000 magnification; 15 kV; (b) ×20 000 magnification; 15 kV.

In order to confirm the mineralogical phase of the CaCO$_3$ microparticles an XRD (X-ray Diffraction) was performed by LNEG. The XRD pattern is consistent with vaterite phase of CaCO$_3$ as show in (Figure 6.6). The calcite phase is also present as proposed by the few low intensity peaks. It is not suppressing to find the presence of some calcite form because this is the most thermodynamically stable phase of CaCO$_3$ (Won et al., 2010).

The characterization of the particles surface was done by SEM. The resultant images show CaCO$_3$ particles that are uniform, nearly spherical with a narrow size distribution (Figure 6.4). The particle size distribution of the suspended particles comprised only one population with an average diameter of 5 μm also confirmed by SEM images (Figure 6.5).
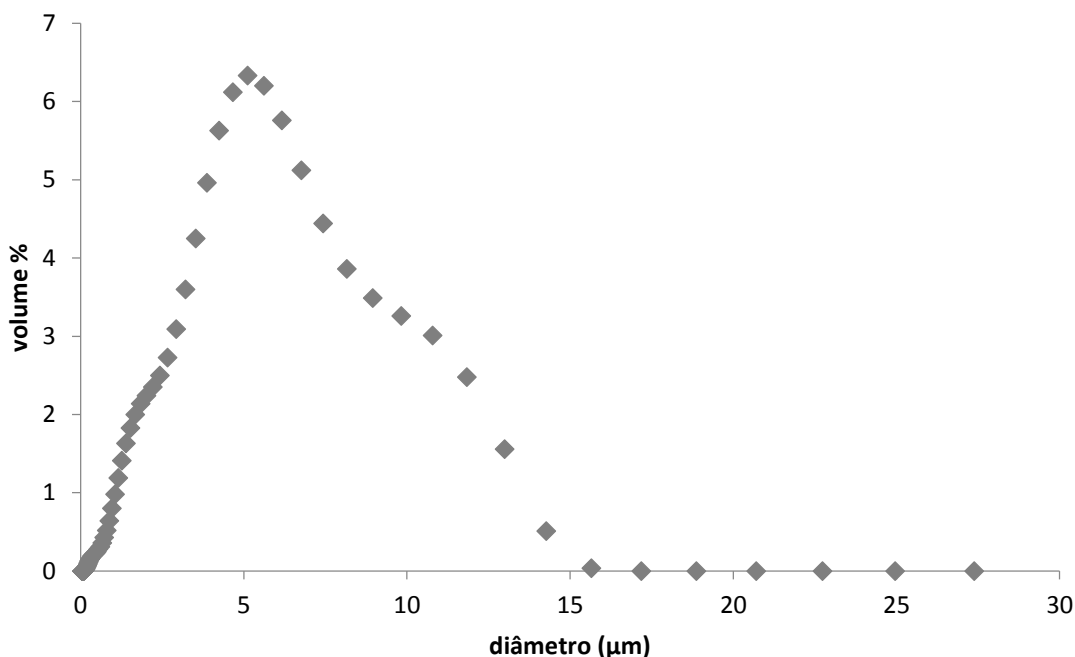
Figure 6.5. Volume distribution of the $CaCO_3$ particles.

HPLC experiments demonstrated that each particle of $CaCO_3$ carried 1.3×10^{-7} mg/L of BDMDAC. The particles of $CaCO_3$ had a zeta potential value of -20 ± 3 mV. During the functionalization process by LbL the zeta potential values of $CaCO_3$-PEI/PSS and $CaCO_3$-PEI/PSS/BDMDAC were -46 ± 4 mV and -18 ± 3 mV, respectively. The significant changes in the zeta potential values after addition of each layer suggest the successful deposition of BDMDAC. PSS is a polyanion inducing high negative zeta potential values (Tedeschi et al., 2004; Volodkin et al., 2004b). The addition of BDMDAC positively charged induced the screening of the charges shifting the zeta potential to less negative values. The hydrophobic interactions between the carbon chains of BDMDAC blocks the access of more BDMDAC molecules to bind electrostatically to the free negative charges of PSS. The final zeta potential of the functionalized particles ensures the stability of the microparticles.
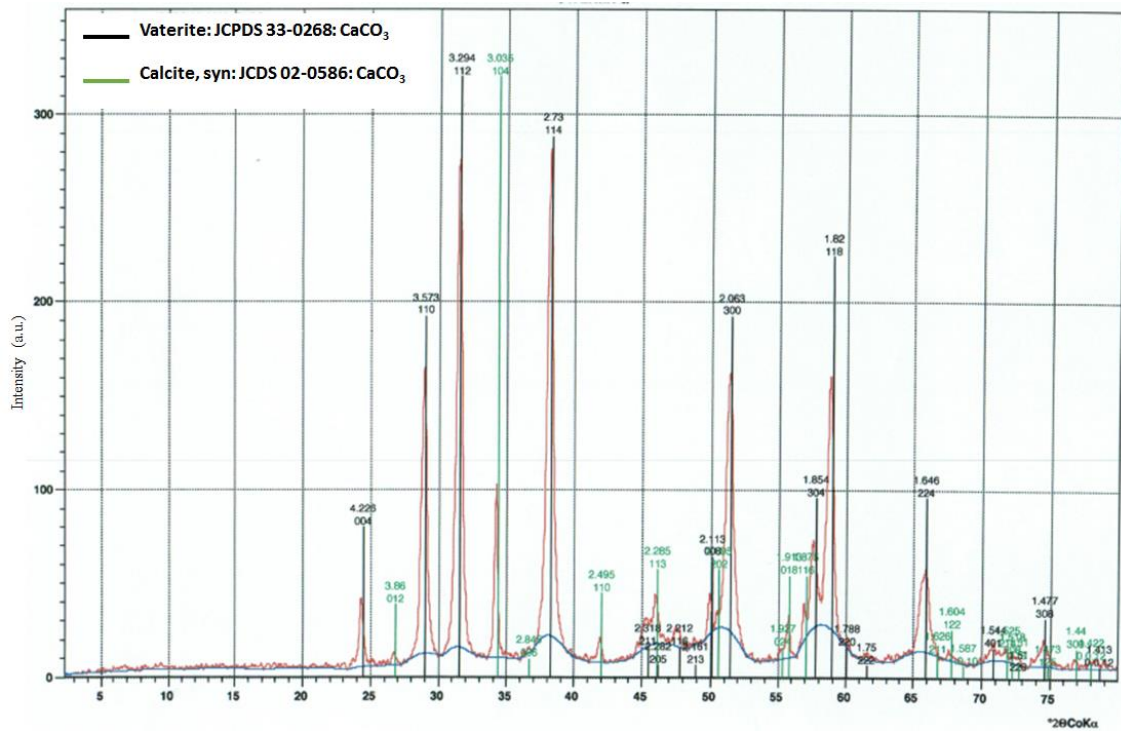
Figure 6.6. XRD pattern of synthesize microparticles of CaCO$_3$

The morphology of biofilm treated with microparticles is different from the other scenarios and it is possible to identify microparticles attached to biofilm (Figure 6.7). The measurement of thickness (Figure 6.8) shows a decrease in the thickness of the biofilms during the treatment with the free and immobilized biocide when compared with the control experiment ($P < 0.05$). The comparison between the effect of free and immobilized biocide on the biofilm thickness shows no significant differences ($P > 0.05$).
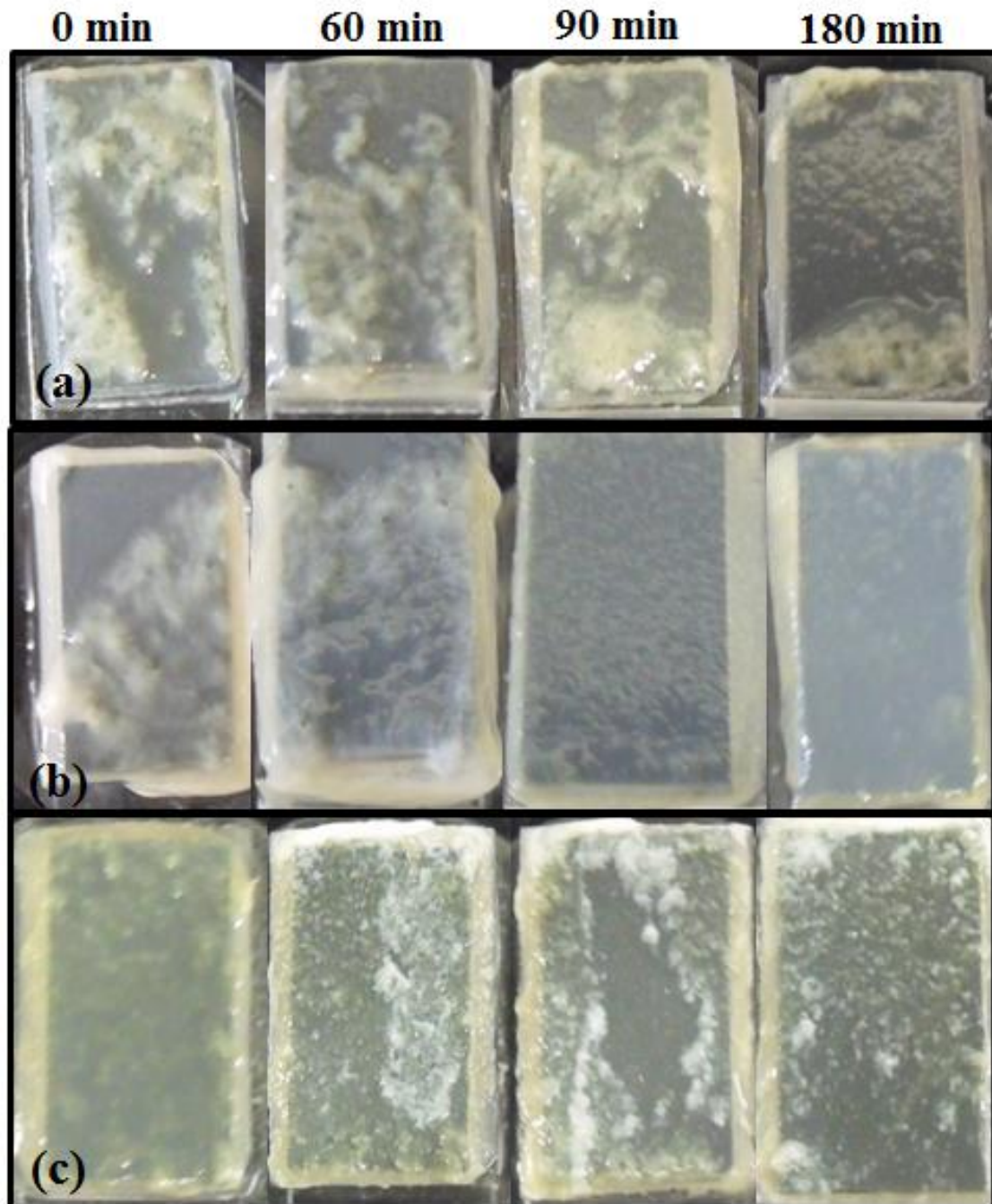
Figure 6.7. PVC coupons with 14 days-old biofilms for the several conditions tested: (a) control experiment (exposure to saline solution); (b) exposure to free biocide; (c) exposure to immobilized biocide.
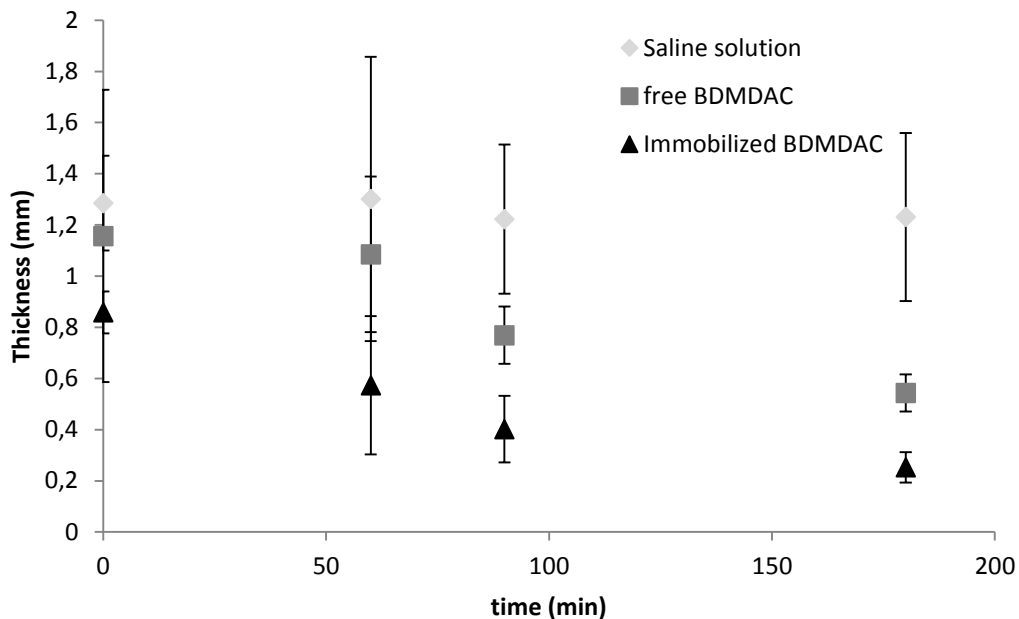
Figure 6.8. Values of biofilm thickness for the several exposure periods. These results are an average of those obtained from two independent experiments.

Additional tests were performed to assess the effects of the biocide on the culturability and viability of the biofilm cells (Figure 6.9 and Figure 6.10). After 60 minutes of exposure there were decreases (1 log reduction) in the numbers of CFU in biofilms treated with both biocide free and immobilized. Once again the difference between the free and immobilized biocide was not significant ($P > 0.05$). Total loss of culturability was detected with a 90 minutes exposure for the free and immobilized biocide.

The results of viability are present in Figure 6.10. The PI component of the Live/Dead BacLight kit only penetrates cells with damaged membrane and these cells are considered dead. The percentage of the non-viable biofilm cells increased considerably with the biocide exposure time for both free and immobilized biocide. For the control experiments the viability of the cells remained constant during the experiment ($P > 0.05$).
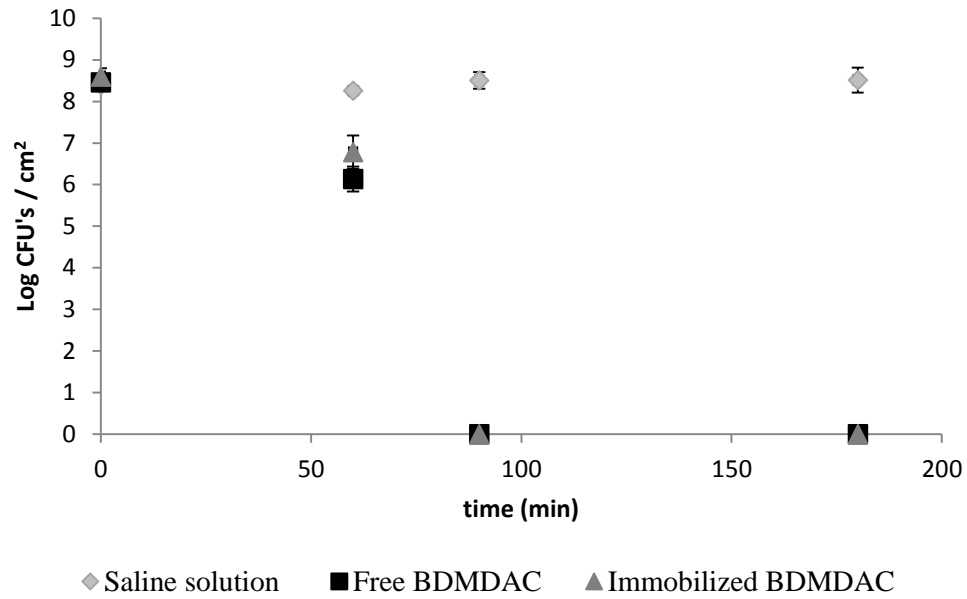
Figure 6.9. Log CFU/cm^2 for the for the several exposure periods. These results are an average of those obtained from two independent experiments.
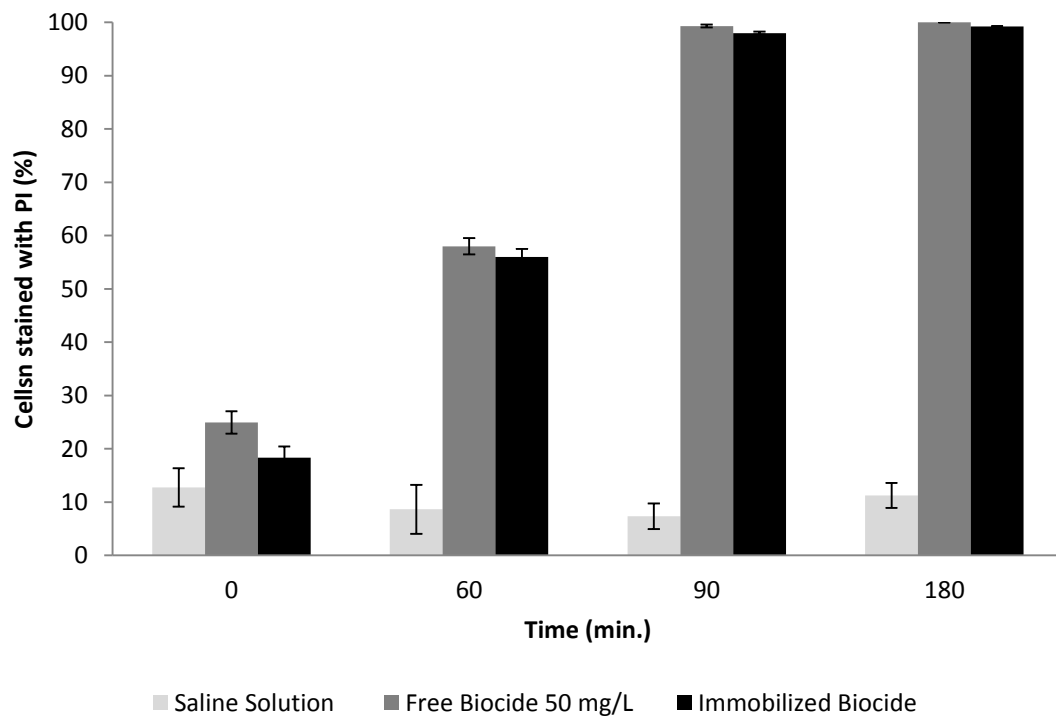
Figure 6.10. The percentage of the biofilm cells stained with PI for the several exposure periods. These results are an average of two independent experiments.

This study with CaCO₃ microparticles carrying the antimicrobial agent demonstrated that this strategy has strong potential to control flow-generated biofilms. Also, the use of immobilized biocide may have a potential public health, environmental and economic benefits by effectively limiting the levels of biocides used in cleaning and disinfection practices.

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Chapter 7

Conclusions remarks and future work

7.1. Conclusions remarks

The goal of this work was to develop and characterize microparticles with functionalized surfaces that act as carriers of antimicrobial molecules and to test them against biofilm formed by *P. fluorescens* in a chemostat and in a flow-cell reactor. It was possible to conclude that the BDMDAC coated particles of PS and $CaCO_3$, even if only partial inactivation was achieved (reduction of viable counts of about 90%), are significantly active against *P. fluorescens* biofilms. $CaCO_3$ coated particles seem to be more active than those of PS, once the characteristic interfacial surface of the $CaCO_3$ allowed an increased amount of BDMDAC in the microparticle.

This promising technology has a potential impact in reducing environmental costs, health risks associated with the intensified use of antimicrobial chemicals, as well as cleaning and disinfection costs in many industrial plants. Studies are in progress in order to assess the life span and the reuse of those active particles.

7.2. Future work

The present work is the beginning of a study that will hopefully have, in the future, a potential impact in reducing environmental costs, health risks associated with the intensified use of antimicrobial chemicals and cleaning and disinfection costs in many industrial plants.

In the future, it would also be of interest to test the efficacy of the developed particles in a larger scale and with different types of microorganisms, particularly against multispecies biofilms. It will be interesting to look at the microparticles as a strategy to prevent microbial contaminations. For example, the microparticles could be tested in a fluidized bed reactor allowing the continuous filtration and killing of microbial contaminants present in the water stream.

Future studies will therefore focus on the use of new particle cores and new biocides, in addition to the ones already tested. It is suggested the use of magnetic materials, such as magnetite. Those will have the advantage of being easier to recover using adequate magnetic fields.