**Integrated Master in Bioengineering**

# Microparticles loaded with benzyldimethyldodecyl ammonium chloride to control adapted resistant bacteria

**Dissertation for Master Degree in Bioengineering**

**Specialization in Biological Engineering**

**Maria Antónia Sousa Correia da Silva**

**Supervisors:**

Idalina Goreti Maia Machado (Ph.D.)

Luís Manuel Ferreira Melo (Full Professor)

U.PORTO

FEUP FACULDADE DE ENGENHARIA
UNIVERSIDADE DO PORTO

**January 2016**

The present thesis was developed for the obtention of the Master degree in Bioengineering, in the Faculty of Engineering of University of Porto.

The work was carried out at LEPABE during 9 months. The main objective of the thesis was to evaluate the antibacterial activity of benzyldimethyldodecyl ammonium chloride loaded microparticles on controlling *Pseudomonas fluorescens* and *Escherichia coli* adapted resistant strains.

The author

_____

(Maria Antónia Sousa Correia da Silva)

The supervisors

_____

(Idalina Goreti Maia Machado)

_____

(Luís Manuel Ferreira Melo)

*'Prestes, larguei a vela*
*E disse adeus ao cais, à paz tolhida.*
*Desmedida,*
*A revolta imensidão*
*Transforma dia a dia a embarcação*
*Numa errante e alada sepultura...*
*Mas corto as ondas sem desanimar.*
*Em qualquer aventura,*
*O que importa é partir, não é chegar.'*

**Miguel Torga, em 'A Viagem'**

# Acknowledgments

A realização desta dissertação não seria possível sem a participação de vários intervenientes, dos quais merecem o meu reconhecimento e gratidão.

Primeiramente, gostaria de agradecer ao meu co-orientador, o Professor Doutor Luís Melo, pela sua orientação científica, compreensão e confiança na realização deste trabalho.

Um obrigada especial à minha orientadora, Drª Idalina Machado, pela ajuda, empenho, disponibilidade com que me direcionou e sobretudo pela paciência com todas as intermináveis questões sobre resistência que foram surgindo ao longo do trabalho. Os seus conselhos foram determinantes para o desenrolar desta dissertação, por isso, o meu obrigada por tudo!

À Ana Meireles, que durante a realização do projeto foi fundamental, por todo o incentivo, amizade e ajuda dada ao longo desta etapa! Por tudo isto e muito mais, expresso a minha gratidão. Obrigada "Catarina"!

Ao laboratório E-007, em particular à Ana Luísa, Anabela, Inês, Joana e Andreia pela simplicidade e recetividade em ajudar que sempre demonstraram.

Ao Departamento de Engenharia Química e ao LEPABE pelas instalações, recursos e financiamento.

Ao Professor Manuel Simões e à Carla Ferreira pela amabilidade em partilhar os seus conhecimentos no que diz respeito à área das micropartículas.

À Paula e Sílvia do E-103 por todo o carinho e alegria que sempre partilharam.

Aos meus colegas da UTAD, por todas as vivências naquela grandiosa cidade de Vila Real e às minhas eternas amigas da U.Católica, Joana, Diana e Prof. Sónia, que contribuíram igualmente para o enriquecimento da pessoa que sou hoje.

Às minhas sempre companheiras, confidentes e acima de tudo por estarem presentes nos bons e menos bons momentos, durante estes 5 anos, especialmente à Dani, Cláudia, Mariana, Ana Maria (mais conhecida por Wow), Morgana e Denise. Obrigada por tudo!

Aos meus colegas de laboratório e amigos André e Pedro pelas gargalhadas e animação 'musical' providenciada desde o pimba ao mais 'requintado' (e'alto-pedigre') *stromae* nas alturas críticas. Ao Duarte, Joana Costa e Diana, esta também é para vocês.

Tenho ainda de agradecer a todos aqueles que se cruzaram comigo e fizeram de mim uma pessoa mais rica e feliz: Soraia, Cristiana, Renata, Suse Francisca, Lisa, Raquel, Vera, Maria João, Dr.ª Mariazinha, D. Custódia, Filipa Lopes 'et' Pierre Bodenés (pour tous les conseils et bonne courage dit!), e à Equipa da Q.B., a todos, o meu agradecimento puro.

Por último, um particular e muito especial obrigada à minha família; Mãe, Pai, Cristina, Nuno e às minhas crianças Sofia e António, por todo o incentivo, paciência, alegria e apoio incondicional durante a realização deste projeto.

Não poderia fechar este rol de agradecimentos, sem incluir a vóvó Alzira, que tenho a certeza que estará a festejar com uma bela chávena de café acompanhada por 'aqueles palitos à Rei'! Esta é pra ti avó Zira!

A todos vocês o meu sincero e profundo OBRIGADA!

# Abstract

The inappropriate and excessive use of antimicrobial agents has led to the development of one of the most preoccupying public health threats, the emergence of resistant bacteria. The ability of microbes to adapt to sub-lethal concentrations of antimicrobials, by genetically or phenotypically ways, contributed to the widespread appearance of novel resistance strains. Considering the types of resistance that are known, it is becoming increasingly recognized that adaptive resistance is one of the major mechanisms behind the acquisition and evolution of drug resistance.

Since antimicrobial agents have lost their effective action against this new era's pathogens, the focus on the development of antimicrobial stewardship strategies and new therapies for microbial resistance control have been outlined in order to try to revert the process. The use of antibacterial microparticles is an example of a new promising strategy, since it can provide a distinct mechanism of action when compared to common antimicrobials.

This work analyses the antibacterial effects of calcium carbonate microparticles coated with quaternary ammonium compound, benzyldimethyldodecyl ammonium chloride (BDMDAC), against major gram-negative bacteria, *Escherichia coli* and *Pseudomonas fluorescens.* These microorganisms were selected since they have high potential for resistance development and cause serious problems in a wide range of areas in the planktonic or biofilm states.

The study of a biocide from the group of quaternary ammonium compounds (QAC's), as BDMDAC, has gained importance since these antimicrobials are extensively used as disinfectants in several environments. In this way, the frequency of exposure coupled with the contact of agents'concentrations, lower than the ones recommended, can conduct to the loss of efficiency of these antimicrobial agents. This inefficiency on killing may promote bacteria adaptation to such concentrations of QAC's. High tolerance of *E. coli* and *P. fluorescens* to QAC's, gained interest since resistance to these compounds caused reduced susceptibility to other classes of antimicrobial agents. The adaptive resistance of *E. coli* and *P. fluorescens* to BDMDAC was investigated and the possibility of cross-resistance development to benzalkonium chloride (BAC) as well as to the antibiotic ciprofloxacin (CIP) was also evaluated. It was shown that *E. coli* exhibited remarkable tolerance to BDMDAC at 40 mg/L and cross-resistance to BAC and CIP. Adaptive resistance of *P. fluorescens* to 45 mg/L of BDMDAC revealed to be unstable and the adapted strain exhibited cross-resistance to BAC.

In this study $CaCO_3$ microparticles (MPs) were developed as a novel antimicrobial agent, through layer-by-layer technique and coated with BDMDAC. The antibacterial activity of BDMDAC MPs was tested against *E. coli* and *P. fluorescens*, wild-type and adapted bacteria. Results showed relevance for resistant strains control, namely of *E. coli* bacteria, since this strain demonstrated higher tolerance to BDMDAC than *P. fluorescens* after de-adaptation process. In this way, it was verified that BDMDAC MPs may turn out as promising technology to control resistant strains.

Overall, the data collected from this thesis revealed that the repeated use of BDMDAC can contribute to antimicrobial resistance development and to the emergence of adapted bacteria, reducing also their susceptibility to other antimicrobials agents. In the future, more studies are needed in order to elucidate the actual risk of the biocide use on generating antimicrobial resistant bacteria, as well as on the efficiency of new MPs as a new antimicrobial agent.

**Keywords** Adaptive resistance; Antimicrobials; Antimicrobial microparticles; Cross-resistance; Layer-by-layer; Quaternary ammonium compounds; Microbial decontamination.

# Resumo

O uso inapropriado e continuado de agentes antimicrobianos conduziu ao desenvolvimento de uma das mais preocupantes ameaças à saúde pública, o surgimento de microrganismos resistentes. A capacidade dos microrganismos se adaptarem após exposição a concentrações sub-letais de agentes antimicrobianos, seja por via genotípica ou fenotípica, tem contribuído para o aparecimento de novas estirpes resistentes. Dos três tipos de resistência estudados até à data, tem-se tornado cada vez mais evidente que a adaptação é um dos mecanismos que melhor esclarece a aquisição e evolução da resistência a produtos antimicrobianos.

Assim, a partir do momento em que se começou a verificar a perda de eficiência no tratamento por parte dos agentes antimicrobianos em uso, a comunidade científica estabeleceu como foco a elaboração de novas estratégias de gestão de antimicrobianos assim como novas soluções terapêuticas, que, ao tentarem reverter o processo de adaptação, irão possibilitar o controlo da resistência microbiana. A utilização de micropartículas antibacterianas (MPs) como agentes antimicrobianos, neste campo de investigação, é reconhecidamente promissora visto que, através delas é possível providenciar distintos mecanismos de ação, que as tornam vantajosas quando comparadas com os produtos antimicrobianos comummente utilizados em estado livre.

A presente dissertação analisou os efeitos antibacterianos de micropartículas de carbonato de cálcio revestidas com o composto quaternário de amónio, cloreto benzildimetildodecilamónio (BDMDAC), testado em bactérias gram-negativas - *Escherichia coli* e *Pseudomonas fluorescens*. As bactérias foram selecionadas, com base no seu elevado potencial de desenvolvimento de resistência tanto no seu estado planctónico como em biofilmes.

O estudo de biocidas pertencentes ao grupo de compostos quaternários de amónio (QAC's), como o BDMDAC, tem adquirido especial importância, uma vez que estes compostos são amplamente utilizados como desinfetantes. Desta forma, a frequência de exposição aliada ao contacto de concentrações de agente inferiores à suficiente para que ocorra morte da população microbiana, poderá levar à perda da sua eficácia. Esta ineficácia na inativação do crescimento bacteriano poderá induzir o desenvolvimento de bactérias resistentes a a essas concentrações de QAC's.

A elevada tolerância da *E. coli* e da *P. fluorescens* a QAC's tem adquirido especial interesse uma vez que a resistência a estes compostos tem-se revelado como possível origem da redução da suscetibilidade a outras classes de agentes antimicrobianos. A indução de resistência adaptativa de *E. coli* e *P. fluorescens* a BDMDAC foi analisada assim como a possibilidade de desenvolvimento de resistência cruzada tanto ao biocida cloreto de benzalcónio (BAC) como ao antibiótico ciprofloxacina (CIP).

O estudo revelou a existência de elevada tolerância de *E. coli* a 40 mg/L de BDMDAC e de ocorrência de resistência cruzada a BAC e CIP. Por outro lado, a adaptação de *P. fluorescens* a 45 mg/L de BDMDAC foi instável, sendo que a estirpe adaptada exibiu resistência cruzada ao BAC.

A fim de se propor um novo agente antimicrobiano, desenvolveram-se micropartículas de $CaCO_3$ segundo a técnica de *layer-by-layer*. Estas MPs foram então revestidas com BDMDAC, como agente antibacteriano. A atividade antibacteriana das MPs de BDMDAC foi testada em *E. coli* e *P. fluorescens* em suspensão de células de coleção e adaptadas. Os resultados obtidos demonstraram ser relevantes quanto ao controlo de estirpes resistentes de *E. coli*, uma vez que a estirpe demonstrou uma maior tolerância às MPs de BDMDAC em relação à bactéria *P. fluorescens* após o processo de desa-adaptação. Deste modo verificou-se que as MPs de

BDMDAC poderiam ser consideradas como possível alternativa como agentes antibacterianos no controlo de estirpes resistentes.

Os dados obtidos no presente trabalho permitiram observar que a utilização frequente de BDMDAC pode contribuir para o desenvolvimento de resistência a antimicrobianos e conduzir ao aparecimento de bactérias adaptadas, diminuindo também a sua suscetibilidade a outros agentes antimicrobianos. Futuramente, será necessário realizar mais estudos no sentido de esclarecer quanto ao risco atual do uso de biocidas no aparecimento de bactérias resistentes assim como relativamente à eficácia das MPs de BDMDAC como novos agentes antimicrobianos.

**Palavras-chave** Resistência adaptativa; Agentes antimicrobianos; Micropartículas antimicrobianas; Resistência cruzada; Técnica *Layer-by-layer*; Compostos quaternários de amónio; Descontaminação microbiana

# CONTENTS

# List of Figures

# List of Tables

# Glossary

*Abbreviations*

**ABR** – Antibiotic Resistance

**ADR** – Adaptive Resistance

**AFSSA** – French Food Safety Agency (*Agence française de sécurité sanitaire des aliments*)

**AMB –** Antimicrobial agent

**AR** – Acquired Resistance

**ATCC** – American Type Culture Collection

**BAC** – Benzalkonium chloride

**BDMDAC** – Benzyldimethyldodecyl ammonium chloride

**$CaCO_3$** – Calcium Carbonate

**CFU/mL** – Colony-forming units per milliliter

**CIP** – Ciprofloxacin

**CLSI** – Clinical and Laboratory Standards Institute

**EUCAST** – European Committee on Antimicrobial Susceptibility Testing

**FDA** – Food and Drug Administration

**GC** – Growth control

**$H_3BO_3$** – Boric acid

**HAI's** – Hospital-acquired infections

**HPLC** – High-performance liquid chromatography

**IR** – Intrinsic resistance

**LBL** – Layer-by-layer

**MBC** – Minimum bactericidal concentration

**$MBC_{adapted}$** – MBC correspondent to the adapted bacteria strains

**MDR** – Multi-drug resistant

**MIC** – Minimum inhibitory concentration

**$MIC_{adapted}$** – MIC correspondent to the adapted bacteria strains

**$MIC_{WT}$** – MIC of wild-type strain bacteria

**MPs –** Microparticles

**NaCl** – Sodium Chloride

**NCCLS** – National Committee for Clinical Laboratory Standards

**NG –** Normal growth

**OD** – Optical density

**OM** – Outer-membrane

**PCA** – Plate count agar

**PEI** – Polyethylenimine

**PSS** – Poly (sodium 4-styrenesulfonate)

**QAC's** – Quaternary ammonium compounds

**SEM** – Scanning electron microscopy

**sub-MIC** – Sub-inhibitory concentrations

**TSB** – Tryptic soy broth

**UP** – Ultra-pure

**WHO** – World Health Organization

**WT** – Wild-type

# Chapter 1

# Work Outline

## 1.1 Background and Project presentation

Antimicrobial agents (AMBs) have saved human life from infections and diseases that were previously fatal. Hence, the use of antimicrobials revealed to have great impact on decreasing morbidity and mortality attributed to these burdens and on giving a renewable hope, ensuring health care for new generations [1]. However, in the last decades, the misuse of such antibacterial agents has provided selective pressure on the microorganisms [2, 3].

In this way, in order to survive to stress conditions, bacteria developed antimicrobial resistance mechanisms, which resulted in the emergence of antimicrobial resistant strains [4, 5]. The spread of bacterial resistance to antimicrobials is a global problem, posing enormous health concerns, including the increased frequency of treatment failures and severity of infections, namely in environments exposed to sub-lethal concentrations of disinfectants [6, 7]. In addition to this problematic, there has been evidences that bacteria insusceptibility to AMBs may develop cross-resistance to other antimicrobials [8].

Facing this era of drug resistance, new approaches are required for designing new antimicrobials agents with more effective and targeted antibacterial action in order to overcome bacterial resistance [9]. The use of antibacterial microparticles has shown to be a promising strategy on the treatment of bacterial contamination on wastewater. Furthermore, it is considered a technique with reduced costs associated with improved product stability [10].

## 1.2 Main Objectives

The main objective of this work was to develop benzyldimethyldodecyl ammonium chloride (BDMDAC) microparticles in order to control the growth of *P. fluorescens* and *E. coli*

previously adapted to the free form of BDMDAC. The cross-resistance of adapted cultures to another biocide, benzalkonium chloride (BAC) and to the antibiotic ciprofloxacin (CIP) was also determined.

*P. fluorescen*s and *E. coli* collection strains were used to assess the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and kill curve patterns in the presence of BDMDAC, BAC and CIP. Both strains were then adapted to increasing concentrations of BDMDAC in order to promote adaptive resistance. MIC at intermediate concentrations of BDMDAC was used to support the adaptation cycles. The kill-curve pattern and MIC of adapted strains were determined in order to verify if BDMDAC adaptive resistance and cross-resistance to BAC and CIP occurred. BDMDAC microparticles were developed in order to assess their antimicrobial efficiency against collection as well as adapted strains.

## 1.3 Thesis Organization

Chapter I describes the main objectives, context and motivations for the development of this work and serves as a guide line to the overall work presented in the further chapters. In addition to this chapter, the work comprises four chapters.

Chapter II is a brief review of the literature. The relevance of scientific research for the development of new antibacterial agents is raised and some properties of agents, like quaternary ammonium compounds, that can be fixed to microparticles as promising drug carriers to control microbial contamination are described. The concern of antimicrobial resistance on bacteria is also focused, as well as the known mechanisms of resistance, from conventional to their derivatives. Owing to the relevance of resistant-bacteria survival after exposure to QAC's sub-lethal concentrations, the bacterial adaptation process is reported. The development of new agents for the control of microbial growth and their antibacterial activity is also reviewed.

Chapter III constitutes a reference to the materials and methods used in the laboratorial studies.

In Chapter IV, obtained results are showed. These include, BDMDAC, BAC and CIP effect against microbial growth in suspension cells of *E. coli* and *P. fluorescens*. The purpose of this work is to evaluate if BDMDAC-$CaCO_3$ microparticles are able to minimize resistance gained by bacteria strains previously adapted to that antibacterial agent. In this topic, the susceptibility of bacteria to BDMDAC, BAC and CIP was evaluated. Furthermore, the ability of gram-negative bacteria to acquire resistance to BDMDAC through an adaptation process was assessed. Subsequently, the study of bacterial growth kinetics in the presence of BAC and CIP, before and after induction of adaptive resistance, is discussed and the cross resistance of adapted strains to these gents evaluated. The microparticles were synthetized and several parameters

characterized, when combined with antibacterial agent – BDMDAC were evaluated in terms of their antibacterial activity against two bacteria (collection and adapted strains). By the end, a predicted scheme is provided, based on data collected from the results obtained as well from background found.

Chapter V gives an overview of the work presented by describing the main conclusions and proposals for future research.

# Chapter II

## Literature Review

In this chapter concepts related with antimicrobial agents (AMBs) are introduced, focusing on aspects like bacteria resistance and the development of new agents for preventing it. Along this section a brief review of existent mechanisms of antimicrobial resistance is presented, with special focus on adaptive resistance and cross-resistance. Finally, a bibliography review of new antibacterial agents as tools to combat bacterial resistance is compiled, highlighting the use of microstructures as drug carriers.

## 2.1 Introduction to antimicrobial agents

The antimicrobial therapy 'golden age' began with the production of penicillin in 1941. Since then, the definition of "antimicrobial" suffered some changes [11, 12]. In the last 70 years, many AMBs have been applied at several fields for the prevention and treatment of microbial contaminations [12, 13].

For the treatment of patients who have infectious diseases, AMBs are defined as therapeutical agents, being designated as antibiotics. As disinfectants, these include a category of "biocidal agents", by following European Union regulations, which are used for controlling and/or eradicating microbes. Regarding the purpose of disinfection, these agents are commonly applied in health care (disinfection of medical devices, surfaces and skin, as antiseptic), in consumer products (from cosmetics to household products), food production (for disinfection of equipment, containers, surfaces or pipework associated with the production, transport and storage of food or drink) and, animal husbandry, providing numerous benefits to society [13-16].

### 2.1.1 Antimicrobial agents

'Antimicrobial' term is predominantly associated to a drug used to treat microbial infections. As a general term, it comprehends a group of drugs that includes antibiotics, antibacterial agents, antifungal and antiviral agents [17]. On one side, the antibiotics define the category of agents that possess the ability to inhibit only a specific physiological process [18, 19]. By contrast, the concept of biocide, that includes antiseptics, disinfectants and/or preservatives, refers to those compounds that appear to have a mechanism of action that operates in multiple sites (within the cell wall, membrane or cytoplasm). The action of these agents is concentration-dependent with subtle effects occurring at low concentrations and more damaging ones at higher concentrations [13].

Antibiotics are defined as natural organic compounds synthesized by living organisms [13]. These comprise any class of organic molecule that inhibits or kills microbes by specific interactions with bacterial targets, without any consideration of the source of the particular compound or class [12]. Most of the antibiotics are employed for the treatment of bacterial infections in humans and animals by being effective in low concentrations, against a limited number of organisms and typically applied on or within living tissues [20].

The term 'biocide' is referred to as a chemical agent with a broad-spectrum in nature, which inhibits or kills microorganisms. Typically, biocides are used against pathogens in suspension or on surfaces. Their efficacy varies considerably depending on the target organism and concentration of each component or synergism among components in different formulations [20, 21].

Nowadays, it becomes crucial to understand the mechanisms of action of these compounds since some studies have shown relations between the emergence of bacterial tolerance to biocides and antibiotic resistance [22].

#### 2.1.1.1 Antibiotics

The word "antibiotic" was firstly introduced by Selman Waksman for describing the discovery of streptomycin in 1943. Two years before, penicillin was the first therapeutic compound to be discovered by Alexander Fleming using the disk-diffusion method for antimicrobial activity determination [12, 23, 24].

Over the years, the concept of antibiotic has been seriously over-interpreted. Antibiotic is normally associated to an agent with a selective action on specific cell targets of bacteria [12].

These compounds often exert their effect through growth inhibition caused by inactivation of a single target and achieve bacterial eradication in conjunction with immune defense mechanisms of the host [17, 19]. In this way, antibiotics can act on one of four bacterial sites: (i) cell membrane, (ii) cell wall synthesis, (iii) protein synthesis and (iv) nucleic acid synthesis [25]. Antibiotic-resistant bacteria that are able to overcome and survive to such mechanisms were found at hospital environment. This resistance of pathogenic bacteria represents a serious clinical issue, since it has been associated to the increasing morbidity and mortality rates, and costs related to the treatment of diseases as well on decontamination [13, 26-28].

***Ciprofloxacin***

Ciprofloxacin (CIP) is a broad-spectrum antibiotic agent of the fluoroquinolone class. Approved by US FDA (Food and Drug Administration, United States) in 1987, CIP has *in vitro* activity against a wide range of gram-negative and gram-positive microorganisms [29-31]. Belonging to the 2$^{nd}$ generation of quinolones, this type of compounds possesses a mechanism of action distinct from other AMBs (like β-lactams, macrolides, tetracyclines, or aminoglycosides) [31, 32]. CIP's bactericidal action results from the inhibition of the enzymes topoisomerase II (DNA gyrase) and topoisomerase IV, which are required for bacterial DNA replication, transcription, repair, strand supercoiling repair, and recombination, thereby inhibiting cell division [31, 33].

CIP can be administrated alone or in combination with other types of drugs for the treatment of infections caused by unidentified pathogens, including urinary tract infections and abdominal infections. CIP activity has been reported to be effective against *P. aeruginosa*, but the acquisition of high-level resistance of this bacteria to fluoroquinolones has limited their utility [34]. In the same way, Naves, Del Prado [35] also showed reduced susceptibility of *E. coli* clinical isolates to CIP.

### 2.1.1.2 Biocides

Biocides are described as owing antiseptic, disinfectant and preservative properties, and have been applied empirically in various ways for centuries. Due to the biocides large range of antimicrobial activity, other concepts may be more precise, including 'bacteriostatic' as agents which inhibit growth and 'bactericidal' for the group of agents that kill the target microbes in suspension or on surfaces [36].

Overall, these products play a critical role in controlling the spread of environmentally transmitted pathogens in health care facilities, at home and in food-processing environments [13, 22]. Biocides are widely used in the food industry for the disinfection of production plants and food containers, for the control of microbial growth in food and drinks, as well as water treatment agents (including drinking water – regulated by Drinking Water Directive 98/83/EC), wastewater treatment and in the decontamination of carcasses [13].

The mechanism of action of these particular agents can be described in three levels of interaction to microorganisms: (i) through outer cellular components; (ii) over the cytoplasmic membrane and (iii) interaction with cytoplasmic constituents [22]. Table 2.1 summarizes the application of different types of disinfectants commonly used in food-processing environments as well in industrial systems and their intrinsic properties [13, 37, 38].

**Table 2.1 - Disinfectant agents commonly used in industrial and food-environments**

| Disinfectant Class | Examples of agents | Applications |
|---|---|---|
| *Biguanides* | Clorehexidine; Polimeric biguanides | Acid/alkaline conditions; applicable to all materials; food contact surfaces; Environmental areas; can/bottle warmers, water treatment; spray, soak, manual, circulation; fog air; Household products |
| *Cationic surfactants* | Quaternary ammonium compounds | Neutral/alkaline conditions; applicable to all materials; Food contact surfaces; Environmental areas/residue can extend activity; mildew control; Water treatment; spray, soak, manual, circulation |
| *Phenolics* | Vanillin; Salicylic acid; Pyrocatechol; Resorcinol; Cresol; Hydroquinone; Eugenol | Lubricants for conveyor lines; water treatment; Household products; Intermediate for industrial synthesis |
| *Substituted phenols* | 1,6-diphenyl-1,3,5-hexatriene (DPH) | Plastics, Explosives, Medicines, Paints, Detergents, Pesticides, Anti-oxidants |

Concerning biocidal properties, biocides can be subdivided in four categories: oxidants, electrophilics, weak acids and cationic membrane-active agents [39]. Among the most commonly used are the chemical groups of quaternary ammonium compounds (QAC), phenols and substituted phenols, and biguanides agents (Table 2.1) [37, 38].

As a result of the increasing apprehension of microbial contamination of everyday living environments, the increased use and misuse of antiseptics and disinfectants, has been confirmed as one of the main causes of antimicrobial resistance development [36, 40]. In this line, further studies are needed to unveil the antimicrobial activity of different types of biocides in order to find a design of new agents with improved effects against resistant-strains [41].

Cationic surfactants (as well defined as membrane-active agents), one of the commonly used group of compound involved in disinfectant formulations, have been selected based on selection and persistence of resistant bacteria [42, 43]. Taking into account their action and numerous applications in everyday living, a deeper research is crucial for the characterization of these agents, since these can possibly induce resistance of pathogens as recently reported [43, 44].

## Quaternary ammonium compounds

Quaternary ammonium compounds (QAC) were firstly obtained from the irreversible and very efficient reaction between tertiary amines and alkyl halides [45]. QAC are cationic detergents, conceptually designed as surfactants or surface-active agents. These molecules have the ability to reduce surface tension and to form micelles, allowing dispersion in a liquid [43]. Figure 2.1 shows the basic structure of QAC.

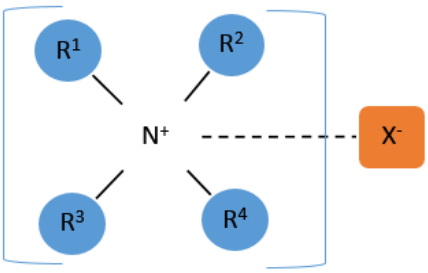



**Figure 2.1 - Basic structure of quaternary ammonium compounds (adapted from Morente, Fernández-Fuentes [22]). The $N^+$ represents the central nitrogen atom; blue circles with $R^1$ to $R^4$ the four aliphatic or aromatic parts (functional groups) and the orange square with $X^-$ the halide.**

The positive charge consists on central nitrogen with four linked groups which occurs in a variety of structures. The anion portion ($X^-$) is commonly established by a chlorine or bromine ion and attached to the nitrogen to form the QAC salt [43]. Thereby, QAC are classified as mono-cationic compounds since these include one quaternary nitrogen with at least one hydrophobic group [46]. QAC such as benzalkonium chloride (BAC), benzyldimethyldodecyl

ammonium chloride (BDMDAC), stearalkonium chloride and cetyltrimethylammonium chloride are well known for their disinfectant properties. Their antimicrobial activity is correlated to the N-alkyl chain length and hence lipophilicity [43, 47].

QAC's mode of action is attributed to their positive charge, which forms an electrostatic bond with negatively charged sites on microbial cell walls [36, 48]. The scenario is supposed to pursue a sequence of events beginning on the adsorption to and diffusion over the cell wall followed by interaction with and disruption of the cytoplasmic membrane causing the release of cellular components and precipitation of cell content and death [49].

Today, QAC's are applied in diverse consumer products and food and health care industries for cleaning, sanitizing, and disinfecting surfaces. Commonly, the formulation practiced in disinfectant product has a concentration ranging 0.03-50% of the total chemical composition [13]. Their low toxicity and ability to be formulated for specific applications and target organisms help account for their widespread usage. However, several recent studies have focused on the potential for the development of resistance among bacterial microbes [43, 50]. Among quaternary ammonium compounds, BAC and BDMDAC are highlighted for being alkyl ammonium agents with considerable usage as disinfectants and antiseptics [51, 52].

***Benzyldimethyldodecyl ammonium chloride***

BDMDAC (Chemical structure in Figure 2.2) represented by molecular formula [$C_{21}H_{38}ClN$], is a QAC with bacteriostatic and bactericidal properties, depending on the concentration used [53]. Concerning physical properties, BDMDAC is a white powder, hygroscopic, stable and incompatible with strong oxidizing agents [54, 55].

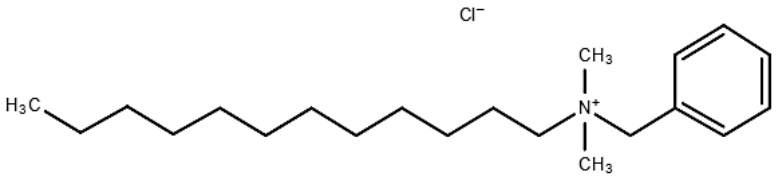



**Figure 2.2 - Chemical structure of BDMDAC (adapted from Ferreira, Pereira [54]).**

The long length alkyl-chain and the positive charge of QAC's are normally correlated with the ability of killing the microorganisms [54, 56, 57]. BDMDAC has been used as cleaning agent with antiseptic, detergent and antimicrobial properties [53].

***Benzalkonium chloride***

BAC, also known as alkyldimethylbenzylammonium chloride, is a cationic surface-acting agent that resulted from a nitrogenous mixture of alkylbenzyldimethylammonium

chlorides of various even-numbered alkyl chain lengths (Royal Society of Chemistry, 2014). BAC is usually used as an antiseptic, having greatest activity when associated with the $C_{12}$–$C_{14}$ alkyl derivatives. This main usage as antiseptic comes from BAC cationic amphiphilic property which confers this product a distinct hydrophobic and hydrophilic region, resulting from nucleophilic substitution of alkyldimethylamine and benzyl chloride [51].

The hydrophilic cationic region destabilizes the microorganism surface by establishing electrostatic interactions with negatively charged cell components. By having the first close contact accomplished through the hydrophilic region, the hydrophobic region of BAC penetrates into the hydrophobic bilayer leading to cell leakage and lysis [36, 51, 58].

In sum, BAC can be used in three distinct applications: as a biocide, as a cationic surfactant and as a phase transfer agent in the chemical industry [59]. Generally, it is found in optical solutions for cleaning applications, minor wound care and disinfection [60, 61]. BAC has demonstrated a broad-spectrum antimicrobial activity over gram-negative bacteria, like *Pseudomonas aeruginosa* and *E. coli*. Moreover, at low concentrations BAC showed to easily induce bacterial adaptation of these microorganisms. *E. coli* biofilms have been found to be resistant to biocides such as BAC and other disinfectants [62, 63].

Studies demonstrated that the biocide presence at low concentrations in the environment, may lead to an increased selective pressure towards disinfectants and antibiotics [13]. Some antimicrobials induce bacterial resistance that confer cross-resistance to antibiotics due to common resistance mechanisms. The efflux pumps and presence of resistance genes for AMBs and antibiotics are some of the mechanisms implied in these situations [36, 64, 65]. It is thus imperative to study resistance mechanisms linked to biocide use and their implication in the potential cross-resistance to other AMBs including antibiotics [61]. Latest studies reported the induction of adaptive resistance, through the incubation of microbes in medium supplemented with sub-lethal concentrations of biocides, and cross-resistance to several related and unrelated antimicrobial compounds [66].

### 2.1.2 Evaluation of bacterial susceptibility

Concentration is one of the most important and determinant factor that controls AMBs efficiency. The clarification of AMB concentration and duration (time) of application determines the efficiency of bacterial growth inhibition, being the design of these parameters of paramount importance when predicting the *in vivo* success or failure of antimicrobial therapy [67, 68].

Bacteria susceptibility is evaluated through the monitoring of the growth response of microorganism after exposure to different concentrations of particular AMB [68]. The protocols

used more often to measure AMB potential are the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) [16, 69]. MIC is the minimal concentration of AMB that inhibits bacteria cells growth and MBC is the effective concentration needed to kill the microorganism [68-70]. In this work, the term bacteria resistance is associated to an alteration by which the AMB becomes ineffective against a microorganism that was previously susceptible to that agent [68].

## 2.2 Biocide usage and resistance development

The widespread usage of AMBs for long time, and sometimes in inappropriate doses, has led to the emergence and development of antimicrobial-resistant microorganisms, that causes the eradication of bacterial contamination more difficult [5, 13, 71, 72]. The huge amount of AMBs as well as the increased strategies acquired by bacteria to evade antibiotics and biocides, have created an enormous impact in resistance mechanisms [5, 25, 73-76].

Confronting this problematic, researchers are continuously working on the development of novel strategies to find a better solution to surpass the problem of resistance development [10, 38].

### 2.2.1 Mechanisms of Antimicrobial Resistance

Over the last years, infections induced through multi-drug resistant (MDR) pathogens became a huge problem in society. The quick dissemination of antibiotics and antibiotic resistance within agriculture community, hospitals, wastewater treatment, and associated environments has been reported and reveals worrying developments [3].

The dimension of this situation has become so large that the World Health Organization (WHO) identified antimicrobial resistance as one of the three most important problems for human health [5, 77]. Resistance has been first reported by the year of 1950 from cationic biocide formulations pointed out as main causes for the selection and persistence of bacterial strains with low-level antibiotic resistance [42].

The bacterial resistance mechanisms are diverse and can be classified as specific or non-specific. In specific mechanisms, the microorganism acts by trying to resist to toxic compounds. In nonspecific mechanisms, resistance is associated to other cellular functions but also exerts a protective effect against antibiotics. Following the same path, resistance can be also reached by mutations that affect the intracellular target for a given antimicrobial drug [28].

In general, bacteria have become resistant to AMB through a range of specific mechanisms of action from: I. Permeability changes in the bacterial cell wall which restricts

antimicrobial access to target sites; II. Active efflux of the antibiotic from the microbial cell; III. Enzymatic modification of the antibiotic; IV. Degradation of the antimicrobial agent; V. Acquisition of alternative metabolic pathways to those inhibited by the drug; VI. Modification of antibiotic targets and VII. Overproduction of the target enzyme [27, 78-81]. Figure 2.3 shows the resistance mechanisms as a cell response to AMBs.

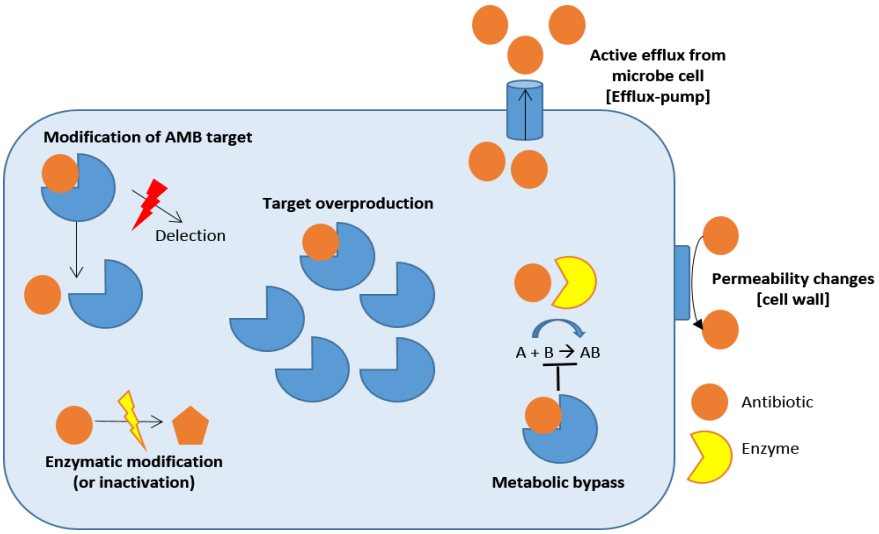



**Figure 2.3 – Bacterial cell and antimicrobial resistance mechanisms (adapted from Schweizer 2012 and SCENIHR 2010).**

The resistance to biocides is known to be mainly a result of the ability of a bacteria to decrease intracellular biocide concentrations below the harmful threshold [13, 82]. Biocides have multiple target sites, thus the emergence of reduced susceptibility caused by modification of target site or through metabolic bypass process is not so common, which in contrast is the more usual resistance mechanism to therapeutic antimicrobials [78, 83].

The development and spread of reduced susceptibility of bacteria can be promoted by several factors. On one hand, the ones inherent to the microorganism like (i) mutation rate, (ii) transmission rate, (iii) biological fitness costs and (iv) the ability to offset for such costs associated. On the other hand, factors related to AMB, in terms of product formulation and its usage which can turn inappropriate if administrated at sub-optimal dosing [68, 84, 85].

In terms of resistance, there are three major types that can be classified as intrinsic, acquired or adaptive resistance [79, 86]. Intrinsic resistance is attributed to a natural property of the microorganism, whereas acquired resistance is due to through forced mutations or acquisition of mobile genetic elements [13]. The concept of adaptive resistance emerged lately and can be defined as the induction of resistance to one or more AMB in response to the presence of a specific signal (environmental stress). This increase generally reverts upon the

removal of a trigger factor and in many situations may lead to non-restoration of the original level of resistance [79].

As new resistance mechanisms are constantly appearing and new genes and vectors of transmission are being identified on a regular basis, it is necessary to know more about them in general in order to classify them properly. Furthermore, investigations have reported that biocides and antibiotics may share common behavior and properties in their respective activity and in the resistance mechanisms developed by bacteria [13].

#### 2.2.1.1 Intrinsic Resistance

Intrinsic resistance (IR) is the type of resistance by which the microorganism phenotype is already defined by the specific ancient phenotype of given bacterial species and also for being common to all members of bacterial species and is inherent to that microorganism [20, 87, 88]. Biocides begin to interact with bacteria at the cell surface, where the mechanism of IR is largely a function of biocide chemical composition and structure of the cell surface [20, 89]. IR mechanisms reported for reducing susceptibility to biocides are essentially linked to the uptake reduction of the agents into the bacteria cell. The mechanisms of action are (i) the permeability barrier of cell envelope, which controls the penetration and adsorption of the agent into the cell, and (ii) the active multi-drug efflux pumps that have the role of mediating drug uptake [78, 90].

Other IR mechanisms are the physiological adaptation and enzymatic transformation of AMB. As result of physiological adaptation, IR is expressed under specific bacteria growth conditions, such as of biofilms state [91, 92]. The poor diffusion of the AMB into the biofilm determines the reduction of susceptibility to that antimicrobial. Also, the chemical interaction between the constituents of the biocidal molecules and the biofilm matrix, and the formation of microenvironments in nutrient- or oxygen-limited, can be on the basis of the reduction of the biocide efficacy [93]. In the case of AMB enzymatic transformation, the occurrence of environmental bio-degradation into various non-lethal compounds has been well-described among microbial communities [94]. Some examples include the enzymatic reduction of the cationic parts of heavy metallic derivatives of silver and copper compounds or the production of formaldehyde dehydrogenase that inactivate aldehydes [94-96].

#### 2.2.1.2 Acquired Resistance

Acquired resistance (AR) is a type of resistance caused by successful gene change and/or exchange that occur after mutations (point mutations, deletions, inversions among others in bacterial genome) or through horizontal and vertical gene acquisition (transfer by

transformation, transduction or conjugation processes). AR is characterized for being inheritable, stable and irreversible [79, 86, 97].

The main mechanisms of AR involve: i) target alteration, ii) impermeability, iii) enzymatic modification or degradation of the compound, and iv) active efflux pumps. As the spectrum of antimicrobial action affects a specific target site within the cell, the acquisition of only one of these mechanisms may lead to bacteria increased resistance to AMBs or to multiple agents within the same class of compounds [83]. Changes in bacterial genome through mutation process or horizontal gene acquisition, may consequently lead to the modification in the nature of proteins expressed by the organism. Such changes may conduct to an alteration in the structural and functional features of the bacteria, which may also result on resistance development against a particular antibiotic [98, 99].

The reduced susceptibility to AMBs is caused by the mutation of genes involved in normal physiological processes and cellular structures from the acquisition of foreign resistance genes or from a combination of these two mechanisms. The simply mutation may change the enzyme or cell structure that consequently changes the affinity or effective activity of the targeted antimicrobials [83]. In the gene acquisition process, the horizontal gene transfer is accomplished through vectors (like plasmids, transposons or integrons) that transfer genes to other microbes of the same specie or to bacteria in another genus or species [83, 98, 99]. In this context, traits of such resistance are then passed on to daughter cells, consequently creating a resistant population which can then spread and be further sources of resistance genes for other strains [100].

#### 2.2.1.3 Adaptive Resistance

From the three types, adaptive resistance (ADR) is the less known and studied [79]. The introduction of this concept emerged by the year of 1971 in a work devoted to *P. aeruginosa* ADR [101]. This was the first study describing the process in which living cells can easily adapt to environmental changes, as result of changes in gene expression triggered through the presence of AMB or to others environmental factors [79]. The study showed that, when conditions are modified, the number of the diverse cell components adjust to the ratios compatible with the optimum rate of growth of the new environment, proving that when growing in stressful conditions bacteria can adapt to that new environment developing resistance [101, 102].

As predominant features, this type of resistance depends on environmental conditions and commonly reverts upon removal of the inducing signal, being transient and not inheritable [79]. The fact of having transient mechanism makes it difficult to detect and in many cases this type of resistance has been disregarded. This phenomenon plays a key role in the differences in

antibiotic resistance observed when comparing *in vitro* with *in vivo* studies and, consequently, it can be involved in clinical failure of some antibiotic administration regimes [79, 103].

In brief overview, the factors that trigger ADR are collected in a group of environmental factors as well as social activities [79]. As environmental traits that influence ADR occurrence, these can be resumed in: anaerobiosis conditions [104, 105], presence of carbon sources [106], cation levels - ions and ionic bonding [107-109], pH variation [110, 111] and polyamines introduction [112-114] . In terms of social behavior, biofilm formation and swarming motility have been related to the increase in virulence as well as in antibiotic resistance and host defense mechanisms [115].

ADR mechanisms are summarized in Figure 2.4 considering the different stimulation conditions [79]. The molecular mechanisms behind ADR have only been recently understood [72, 103, 116, 117]. Results showed that these mechanisms are more complex than initially thought, involving intricate regulatory responses [79].

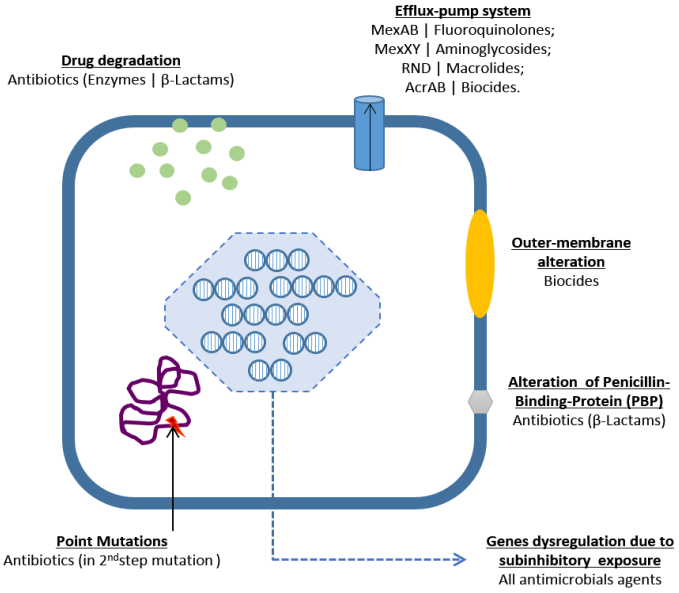



**Figure 2.4 – Representation of some of the major known ADR mechanisms (adapted from Fernandez, Breidenstein [79]). Initials RND stand for resistance nodulation-cell division type efflux.**

In most of topics related to adaptive resistance, more studies need to be carried out in order to characterize the effect of these cues and evaluate their significance regarding the enormous interest on development antibacterial agents with higher efficacy against resistant bacteria.

### 2.2.2 Cross-Resistance

Over the years, microorganisms have acquired mechanisms by which can become less susceptible to the action of different biocides. Nonetheless, these mechanisms have offered a

considerable reduction in the susceptibility to these compounds. The improper use of certain active substances like biocides in several settings may have contributed to the increased occurrence of antibiotic-resistant bacteria [118]. Figure 2.5 are summarized some mechanisms that may have contributed to the development of antimicrobial resistance phenomenon.

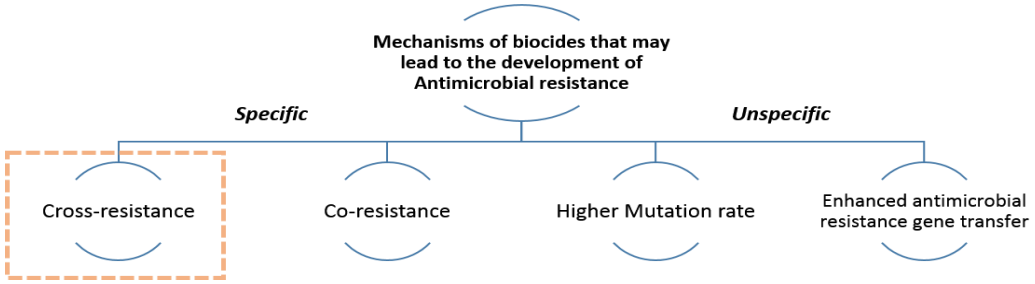



**Figure 2.5 – Possible mechanisms by which biocides may had led to the emergence of antimicrobial resistance (adapted from Seier-Petersen et al. [119]).**

The occurrence of cross-resistance between biocides and antibiotics and, between different antibiotics, has been reported for some time, suggesting that if two antimicrobial compounds have similarity in their mechanisms of action against bacteria, they may also share resistance mechanisms [120, 121]. This process was also reported with potential to occur when different antimicrobial agents act on the same target and initiate a common pathway to cell death, or share a common route of access to their respective targets [122].

## 2.3 New antimicrobials agents

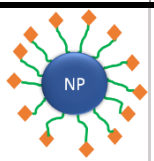
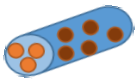
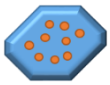
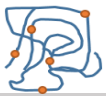
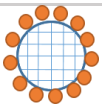
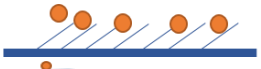
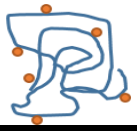
As a response to the challenge of the new era of antimicrobial resistance, recent studies have been developed in order to study new platforms for efficient AMB delivery. Proposals for candidates to antimicrobial therapy have emerged from the use of enzymes to nucleic acid therapeutics, as well from using phage therapy [123], from plant-derived compounds - phytochemicals [124], the development of biocidal polymers from cationic polymers to antimicrobial peptides and their engineered mimetics [125, 126] and also through the design of nano- and micro-structures with antimicrobial activity [10, 127, 128]. In light of the recent scientific reports, these approaches suggested the use of antimicrobial micro- and nano-materials as an alternative to conventional AMBs by which microorganisms may not be able to develop resistance [129-131].

The concept of microparticle (MP) is the designation used for structures with usually spherical configuration and diameters in the micrometer range, normally from 1-1000 µm. Antimicrobial polymeric microparticles are generally formed by a polymer matrix in which a small amount of an active compound can be immobilized [128].

**Microparticles loaded with benzyldimethyldodecyl ammonium chloride to control adapted resistant bacteria**

MPs can be manufactured from a large variety of materials, both natural and synthetic, and by many different preparation techniques [132]. Both starting materials and preparation techniques allow the preparation of an enormous variety of MPs, in terms of size, distribution, composition, surface chemistry, topography and morphology [132]. Specifically, antibacterial particles can be composed of metals, metal oxides, metal salts, metal hydroxides, organic carriers loaded with antibacterial agents, hybrid materials and polymers exhibiting antibacterial properties [10]. Table 2.2 represents some examples of antibacterial microparticles used in two possible forms, dispersed in water or immobilized.

**Table 2.2 – Distinct ways of antibacterial microparticles occurrence (adapted from Moritz and Geszke-Moritz [10]). Orange represents the presence of the antibacterial agent; NP stands for nanoparticle**

| Form of antibacterial agent dispersion | Agent form on particle | Matrix of incorporation |
|---|---|---|
| **Water-dispersed** | Functionalized     Non-altered Stabilized   | Dispersed in the solution |
| **Immobilized** | Incorporated inside matrix | Nanotubes   Matrix lump Fibers  |
| | Installed at surface |  Particles Modified surface   Fibers |

Antimicrobial microstructures can be produced in many distinctive forms depending on the desired application [128]. The antimicrobial agents can be presented through a stabilized form at host-structure, functionalized, as well at structure surface or having a non-altered

antimicrobial structure. Associated to membranes/matrix, antibacterial agents appeared to be incorporated in nano- (scale) materials, like carbon nanotubes or graphene oxide, for example, as well as inside hydrogels and films with chitosan, agar or calcium alginate compounds [10, 128].

In others cases, AMB's can be also incorporated in metal structure surfaces or in mineral and inorganic materials. Some examples are silver, copper or silica particles as well minerals like hydroxypatite, or even of biolymers incorporated in natural fibers like cotton, silk, collagen or cellulose [10].

As described previously (table 2.2), the antibacterial agents can be incorporated in several ways, affecting the agent release efficiency. In terms of drug delivery at the target site, the polymeric drug carriers can release the agent by three general physicochemical mechanisms: i) the swelling of the polymer particles through hydration followed by release through diffusion; ii) enzymatic reaction that results in rupture or cleavage or degradation of the polymer at site of delivery, releasing the drug from the entrapped inner core; iii) dissociation of the drug from the polymer and its deadsorption/release from the swelled particles [133].

***Advantages and drawbacks of MP usage***

MPs in comparison to the use of AMB in free form, offer many distinctive advantages for medical and disinfection purposes [134]. The application of these polymeric microstructures provides: (i) a sustainable and controlled release of the AMB; (ii) a better stability of the AMB; (iii) an increase of bioavailability of agent transport; (iv) the application over specific targets; (v) lower cost; (vi) reduction of the AMB dosage and toxicity [10, 129, 135]. However, its use also implies some concerns. Factors like the large particle size, compared to alternative additives, can result, for instance, in surface texture or gloss reduction or the stability of the encapsulated material during processing which may present additional challenges [128]. Reports have revealed some drawbacks on the application of such MPs [136]. Obviously, for each situation of MPs use, different limitations might emerge related with the material properties such as surface chemistry, particle size, and, also with the biological effect and abiotic processes on the surrounding environment [137, 138].

Regarding the toxic potential of the application of MPs, some studies referred as problematic: i) their destiny in the environment, depending on their material properties, ii) the suspension as individual particles, iii) the formation of aggregates or iv) the, dissolution, or reaction with natural materials [139]. Other fact reported were that, due to their small size and slower rate of gravitational settling, some MPs may remain suspended in air and water for long time periods and may be readily carried over much greater distances than larger particles of the same material [140]. Lubick et al. [141] referred to that the surface chemistry and therefore the mobility of microstructures in porous media, could be affected by the addition of surface

coatings. For example, $TiO_2$ can be harmless in soil, but can be of concern in water once a surface coating is added. Another study revealed that many MPs containing inherently non-biodegradable inorganic chemicals associated to such as metals and metal oxides may not be biodegrade [140].

## Techniques for Microparticles production

During MPs preparation, the selection of the optimal method is crucial for the efficient entrapment of the active substance into the core surface. There are several pharmaceutically acceptable techniques for MPs preparation [135]. For this study the functionalized method (interfacial polymerization) was selected since this technique has shown reliable results for the same purposes [38]. The specific layer-by-layer self-assembly (LBL) technique was used for the development of functionalized microparticles.

*Layer-by-layer (LBL) Technique*

Microparticles obtained by LBL are an emerging class of therapeutic agents that allow the control over crucial parameters that aids on improved drug and carrier pharmacokinetics as well as on enhancing molecular-targeting capabilities [142].

The LBL technique uses electrostatic attraction and complex formation between polyanions and polycations to structure supramolecular multilayer assemblies of polyelectrolytes [143, 144]. The first stage of antimicrobial microparticles fabrication involves the step-wise deposition of polyelectrolytes from aqueous solutions. The polyelectrolyte multilayer film is formed by the alternate adsorption of oppositely charged layers into the particle. After each adsorption step, the non-adsorbed polyelectrolyte in solution is removed by repeated centrifugation or filtration and centrifugation steps [145, 146]. The original method was introduced in 1991 by Decher and co-workers for the construction of pure polymer multilayer films on planar supports [147].

In terms of MPs composition, porous calcium carbonate ($CaCO_3$) has been reported as being an effective core for the development of biocompatible composite materials as result of its availability in nature as a biomineral and, also its porous structure that is suitable for AMBs loading. There are several studies that reported the use of $CaCO_3$ as drug carrier, for encapsulation of proteins and, for biosensing [148-150].

### 2.3.1 Microparticles Applications

Microparticles have been successfully applied in several fields. Due to their promising properties, MPs global market is predicted to increase from 2010 to 2015, in fields as industry

on life science as well as on medical technology [151]. In the pharmaceutical field, MPs are used as structures-based drug delivery systems agents in the treatment of chronic diseases [152, 153]. Also, besides their action in therapeutical settings, MPs are a successful tool as fungicide agent on paints and coatings [154], as food preservatives [155], as AMB present in burn dressings [156], in safe cosmetics [157] and health care [158], in water treatment [159, 160], as well as antimicrobial coatings in medical devices [161, 162] and as disinfection agents in clinical and industrial settings [130, 163, 164].

### 2.3.2 Antibacterial activity

With the introduction of MPs it is necessary to be aware of the mechanisms that confer them antibacterial activity. This may cause cell wall or membrane damage or detrimental changes in cellular organelles [10]. Generally, MPs show good antibacterial properties due to the large surface area to volume ratio that contacts with the bacterial cell [165]. The quality of MPs core was found to be strongly dependent on several factors such as the type of the salts used, the concentration applied, pH, temperature, rate of mixing the solutions and also, agitation level of the reaction mixture [166]. As it was mentioned in the previous section, MPs are frequently used as antibacterial drug carriers. These are nano-based formulations of several antimicrobial drugs that have shown improved pharmacokinetics and antibacterial efficacy by achieving a sustained release directly at the target site [167]. Nonetheless, some of these predicted mechanisms are speculative, so, more studies in this field are needed [10].

In Table 2.3 are resumed some studies that evaluated micro and nanoparticle action in several applications.

**Table 2.3 - Description, background and cost estimation of possible cores for microparticles for the use in several needs**

| Antibacterial activity | Drug carrier | Cores | Size (µm) | Price (€)/g | Techniques/Procedure | Applications | Reference |
|---|---|---|---|---|---|---|---|
| *Streptococcus salivarius*<br>*Streptococcus sanguinis*<br>*Streptococcus mitis*<br>*Candida albicans* | Ethanol extract of propolis | Gelatin | 2.27 - 2.48 | 0.97 [a] | Spray-drying technique | Medical (Oral) | [168] |
| *Listeria ivanovii* | Magainin-I | Si and paramagnetic Si | 5.2-5.4 | 23.92 [a,1] | Silanation protocol (Surfaces Polymerization) | Medical, Cosmetic, and Biomedical fields | [169] |
| *Escherichia coli*<br>*Salmonella typhimurium* | Copper | $Cu^{2+}$ | 50 - 100 | 4.32 [a] | Ball milling method | Disinfection | [170] |
| *Pseudomonas fluorescens* | BDMDAC | $CaCO_3$ | 2 - 4 | 0.11 [a] | LBL Technique | | [171] |
| ----- | Ibuprofen, Nifedipine, Losartan potassium, and Metronidazole benzoate | | 17.9 | | Solvent evaporation | Medical (Oral) | [172] |
| | Doxorubicin | PLGA | 0.125 | 208.50 [a] | LBL Technique | Biomedical field | [142] |
| *Staphylococcus aureus*<br>*E coli*<br>*C. albicans*<br>*Enterococcus faecalis* | Thyme oil \| Glutaraldehyde | Chitosan | 4.71 ± 1.42;<br>13.65 ± 4.34 | 1.58 [a] | Emulsion by cross-linking method | Antimicrobial therapy | [173] |
| *E. coli O157:H7* | Chitosan | | 0.43 - 0.86 | | | | [174] |
| *Bacillus cereus*<br>*E. faecalis*<br>*S. aureus*<br>*E. coli O157:H7*<br>*S. typhimurium*<br>*Salmonella enteritidis* | Selenium dioxide ($SeO_2$) | Selenium | 0.01 - 0.05 | 8.52 [a] | Synthesis by reduction of aqueous Se-ion using the growth culture supernatants of *Bacillus licheniformis* | Antibacterial, Antiviral, and antioxidant therapy | [175] |
| *S. aureus and E. coli* | Catechin | Catechin-Cu(II) | 0.0053 ± 0.0001 | 14.40 [a,b] | Ultrasonic cell crushing method | Antibacterial activity | [176] |

Notes: [a] – Price suggested by *Sigma-Aldrich*; [b] – Final price was estimated based on sum of prices required from each core components; [1] – Silica particles with 5 µm.

Antimicrobial structures can be designed through an extensive list of methods. Their development may involve the application of different materials that include: i) metal compounds for example, silica, silver as well copper; the use of ii) polymers like poly (lactic-co-glycolic acid) (PLGA), chitosan or even gelatin; or also of, iii) organic and inorganic compounds, as suggested the calcium carbonate ($CaCO_3$) composite material.

Facing some of mentioned techniques, for the present study, the studies performed by Ferreira, Pereira [171] and Sato, Seno [150] were used as basis for the development of functionalized microstructures for the desirable applications defined in the work objectives. In this context, this work aims to verify the use of microparticles as antimicrobial solution against *E. coli* and *P. fluorescens* that have gained adaptive resistance to BDMDAC.

# Chapter III

## Material and Methods

In this chapter the analytical and instrumental methods used throughout the experimental work are described. The rationale beyond its employment is also discussed.

## 3.1 Bacteria strains and growth conditions

*Pseudomonas fluorescens* (ATCC 13525) and *Escherichia coli* (CECT 434) used in the study were purchased from American Type Culture Collection (ATCC) and Spanish Type Culture Collection (CECT). The selection was done considering the high intrinsic resistance of such bacterium to biocides in food industry, hospitals as well as in water and wastewater treatment systems [48, 177-181]. Both bacteria were cultured on Plate Count Agar (PCA; *Merck*, Portugal) and incubated for 24 h at 27 ± 3 ºC. The cultures were grown in 100 mL flasks with 25 mL of Tryptic Soy Broth (TSB; *Merck*, Germany), overnight at 37 °C in agitation conditions (120 rpm; Agitorb 200 ICP, *Norconcessus*, Portugal).

## 3.2 Antimicrobial agents

The antimicrobial agents benzyldimethyldodecyl ammonium chloride - BDMDAC (*Fluka*, USA), benzalkonium chloride - BAC (*Sigma Aldrich*, USA) were used throughout this work. The antibiotic ciprofloxacin - CIP (*Sigma Aldrich*, USA) was also used. Stock-solutions of biocide agents were prepared at final concentrations of 1000 mg/L (BDMDAC and BAC) and 60 mg/L (CIP) in distilled water. Diluted solutions were prepared from the stock solutions in order to range the concentrations needed for the subsequent experiments.

## 3.3 Antimicrobial activity

### 3.3.1 Determination of the bacterial growth kinetics

In order to assess the growth pattern of bacteria in the presence of antimicrobial agents, the kill curves were determined at different antimicrobial concentrations. One colony of each bacterium was collected from the PCA plates and grown overnight in Tryptic Soy Broth (TSB, autoclaved at 121 ºC for 20 min). Bacteria were washed by centrifuging the culture (Centrifuge 5810R, *Eppendorf*, Germany) at 4000 rpm (16800g), 15 min, being the supernatant discarded and the pellet, containing bacterial cells washed twice with sterile saline solution (0.85% NaCl, *Sigma Aldrich*). Final suspensions were prepared by adjusting the optical density at 610 nm ($OD_{610nm}$), using V-1200 Spectrophotometer (*VWR®*, Pennsylvania), to 0.200±0.05 (corresponding to $1.0x10^8$ cells/mL) in TSB. In a microtiter polystyrene (PS) 96-well round tissue culture plate (*Orange Scientific*, Belgium) containing 100 µL of broth supplemented with BDMDAC (final concentration ranging from 0.8 to 100 mg/L), were inoculated 100 µL of bacteria cells in TSB. Each plate included negative and positive control. The $OD_{610}$ of each well

content was recorded using an automated plate reader (ELISA™ SynergyTMHT, *BioTek*, USA) as a measure of bacterial growth. The average $OD_{610}$ and the standard deviation from eight different wells were determined for each time point and each condition tested. This experiment was performed in three independent assays.

### 3.3.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal activity (MBC)

The MIC and MBC of antimicrobial agents of *P. fluorescens* and *E. coli* were determined by the microdilution method according to Clinical and Laboratory Standards Institute [182]. Briefly, 96-well round-bottom polystyrene (PS) microtiter plates (Orange) with a total well capacity of 300 µL were used. In each well, 100 µL of fresh TSB with increasing concentrations of AMBs were added to 100 µL of each bacterial inoculum (containing 1 x $10^8$ cfu/mL). The culture plates were incubated at 37°C for 24 h in an orbital shaker at 120 rpm. The highest concentration of BDMDAC that did not promote growth was recorded as the MIC. This MIC-value defines essentially the minimal concentration of AMB that inhibits bacteria cells growth. The MBC was then determined by plating onto PCA a volume of 10 µl of the bacterial suspension where no growth was observed. Those were incubated for 24 h at 37°C. The product concentration, were no colony growth was observed was recorded as the MBC.

## 3.4 Adaptive resistance

### 3.4.1 Adaptation of bacteria to BDMDAC

Adaptive resistance was induced by subculturing bacteria in TSB supplemented with increasing BDMDAC concentrations, according the adaptive procedures described by Machado, Coquet [183]. Five milliliters of an overnight culture (1 × $10^8$ CFU/mL) were added to flasks containing 60 mL of TSB supplemented with BDMDAC at final concentrations starting from 24 to 40 mg/L, (concentration increasing 2 mg/L between passages), and from 30 to 45 mg/L, (concentration increasing 5 mg/L between passages), for *E. coli* and *P. fluorescens* respectively. Cultures were then incubated at 37°C for 24h on a horizontal shaker (120 rpm). Bacterial growth was monitored by OD measurement at 610 nm ($OD_{610}$). Every period of 10 h, 10 mL of the bacterial culture, supplemented with the highest BDMDAC concentration for which bacterial growth was observed, was used to inoculate 60 mL of TSB containing BDMDAC in a final concentration higher than the one that exhibited growth.

At the end of the eighth growth cycle for *E. coli* and the third growth cycle for *P. fluorescens* in increasing concentrations BDMDAC solutions, no significant bacterial growth

was observed. Bacteria were then subculture in the presence of the maximum BDMDAC concentration that allowed growth for same number of complete cycles. To preserve the BDMDAC-adapted strains, petri dishes were prepared with PCA supplemented with BDMDAC at a final concentration of 40 and 45 mg/L (referred as adapted-strains of two bacteria as *P. fluorescens* A45 and *E. coli* A40, respectively). MIC increase factor for each antimicrobial product was determined for each strain as a ratio between MIC after adaptation and MIC before adaptation procedure. The adaptation procedures were carry out in three independent replicates.

***Adaptive resistance stability***

The stability of the BDMDAC-adapted strains was determined by continuous subculturing (every 24 h) for 10 passages in TSB and evaluation of the de-adapted strains ability to maintain their growth in PCA supplemented with BDMDAC. The MIC of the de-adapted cultures was also determined for each strain in three independent replicates.

***Cross-resistance evaluation***

The possibility of cross-resistance occurrence between the different classes of antimicrobials, namely between the BDMDAC-adapted strains resistance to BAC and CIP was assessed. The method used for evaluation consisted on the determination of MIC and MBC of the adapted strains as well as the kill curves pattern in the presence of concentrations of BAC ranged from 1.6 to 200.0 mg/L and CIP from 0.05 to 3.00 mg/L.

## 3.5 Microparticles as novel antimicrobial agents

### 3.5.1 Calcium carbonate microparticles formation

Calcium carbonate ($CaCO_3$) spherical core particles were obtained through the procedure described by Ferreira, Pereira [54]. The oppositely charged electrolytes, Polyethyleneimine (PEI; *Fluka*, USA), Poly (sodium 4-styrenesulfonate) (PSS; *Sigma Aldrich*, Denmark) were assembled on $CaCO_3$ cores, in a process that comprised three steps [184]. The particles were prepared using the layer-by-layer (LBL) self-assembly technique. The original method was introduced by Decher [143] for the construction of pure polymer multilayer films on planar supports.

Firstly, $CaCO_3$-cores were prepared by adding 25 mL of 0.33 M calcium chloride (*Sigma Aldrich*, Denmark) and 25 mL of 0.33 M sodium bicarbonate (*Sigma Aldrich*, Denmark) solutions prepared in distilled water. This mixture was shaken at 1000 rpm during 30 seconds at room temperature. The final solution was placed at room temperature during 5 minutes without

agitation, being after centrifuged at 4000 rpm (16800g), during 10 min. The resulting pellet was resuspended in ultra-pure (UP) water. This washing procedure was repeated 3 times with UP water. The last step of washing was done with 99% ethanol. Obtained microparticles were let to dry during 1 h in desiccator being after stored at room temperature.

### 3.5.2 Microparticules characterization

The microparticles core obtained in the previously described method were first characterized by scanning electron microscopy (SEM) to analyze particle size and configuration before and after the coating process. The SEM equipment of CEMUP (FCUP, Portugal) allowed the observation of the sample in its natural hydrated state. SEM / EDS exam was performed using a High resolution (Schottky) Environmental Scanning Electron Microscope with X-Ray Microanalysis and Electron Backscattered Diffraction analysis: Quanta 400 FEG ESEM / EDAX Genesis X4M. The sample was fixed on a holder with a layer of carbon-rich conductive glue. Then, the sample was coated with an Au/Pd thin conductive film during 60 sec. The sample was further placed into the observation chamber with a rod.

### 3.5.3 Antimicrobial Coating process

$CaCO_3$ microparticles were coated with BDMDAC as described in Ferreira, Pereira [171] and schematically represented in Figure 3.1.

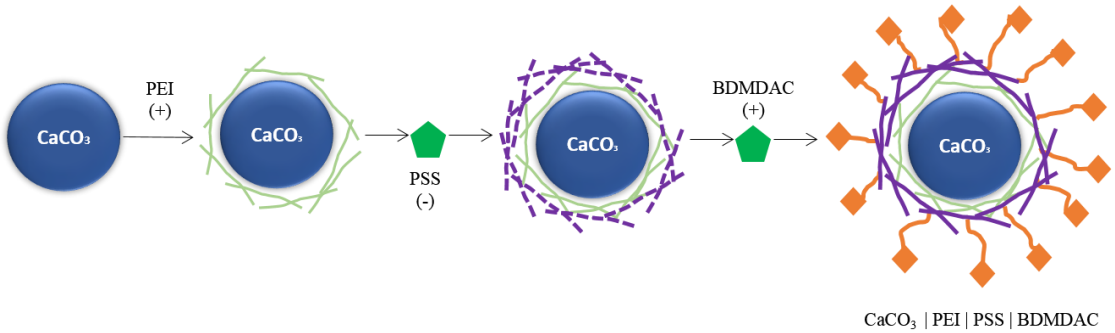



**Figure 3.1 – Process of layer-by-layer technique applied on calcium carbonate ($CaCO_3$) microparticles adapted from Ferreira, Pereira [171] . The green pentagon represents a centrifugation and washing step, green lines – Polyethylenimine (PEI); purple lines – Poly (sodium 4-styrenesulfonate) (PSS) and orange – Benzyldimethyldodecyl ammonium chloride (BDMDAC).**

The $CaCO_3$ particles (in powder form) prepared as described previously, were suspended in an aqueous solution with a PEI solution 10% (w/v in 0.1 M borate buffer) for 20 min being after washed twice in 0.1 M borate buffer (*Sigma Aldrich*, USA) solution, to remove excess PEI. The resulting microparticles (positively coated with PEI) were after coated with PSS (0.1 M borate buffer) during 20 minutes, being after washed with 0.1 M borate ($H_3BO_3$) buffer [171]. To remove the excess polymer, the suspension solution with PSS was

washed. Finally, BDMDAC solution (1000 mg/L in $H_3BO_3$) was added and this solution was let to react during 24 h. After, the solution was centrifuged at 4000 rpm (16800g) for 2 minutes (in order to avoid the particles aggregation) and the pellet containing the coated microparticles was suspended in 0.1 M $H_3BO_3$ solution at pH 9 and kept in cold conditions (4°C). BDMDAC concentration at microparticle surface was previously defined as 300 mg of BDMDAC per liter [171]. This concentration was defined as the theoretical value and was further used as reference concentration.

### 3.5.4 Microparticles antimicrobial activity

The evaluation of the antimicrobial activity of the synthesized microparticles was assessed through the determination of colony forming units (CFU) of the microbial population after exposure to different concentrations of BDMDAC-coated microparticles. Overnight cell suspension obtained as described in section 3.1, were washed and the number of cells was adjusted to 0.200±0.05 ($OD_{610}$, $1.0x10^8$ cells/mL) in TSB. BDMDAC coated microparticles were used to prepare antimicrobial solutions in final concentrations ranging from 1/2 MIC to 4xMIC (BDMDAC MIC determined for each collection and adapted strains with BDMDAC free form). Briefly, 100 mL flasks containing 50 mL of TSB supplemented with BDMDAC microparticles and bacteria cultures were incubated at 37 °C during 24 h on a horizontal shaker (120 rpm). Bacterial growth was monitored hourly by plating and counting CFUs from each concentration tested through the method of spread plating on PCA after serial dilutions.

## 3.6 Statistical Analysis

All experiments were carried out in triplicate. The results were presented as the Mean ± SD (standard deviation of the mean).

# Chapter IV

## Results and Discussion

The results obtained are described and discussed in the present chapter.

**Microparticles loaded with benzyldimethyldodecyl ammonium chloride to control adapted resistant bacteria**

## 4.1 Introduction

Biocides are widely used in several environments from food-industry to hospitals sometimes in inappropriate dosages. Bacteria are constantly exposed to stress environments as well as to sub-inhibitory concentrations of such compounds, this event being referred to as a possible cause of antimicrobial resistance emergence and development [44, 79, 86].

The expansion of biocide usage is predicted to continue, therefore the hazard of biocide use leading to the selection of drug-resistant bacteria, followed by the selection and dissemination of resistant strains, is of rising concern [121, 185, 186]. Extension of this phenomenon had such impact on the environment that led to the urgency on creating novel ways to delay the appearance of new resistant strains [13]. Such approaches have been based on the development of new ways of delivering traditional antimicrobial agents in a more efficient way [129].

The purpose of this work was the development of microparticles carrying benzyldimethyldodecyl ammonium chloride (BDMDAC) to impair the growth of *P. fluorescens* and *E. coli*. The antimicrobial effect of those MPs on the growth of strains previously adapted to BDMDAC was also determined.

## 4.2 Bacteria susceptibility to antimicrobial agents

Antimicrobial susceptibility tests provide relevant information on the prediction of *in vivo* efficient action or failure of certain antimicrobial treatments [16]. For the evaluation of microorganisms susceptibility to the antimicrobial agents, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined. The data of MIC- and MBC-values of antibacterial agents for the microorganisms studied are summarized in Table 4.1.

**Table 4.1 – Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of MIC of benzyldimethyldodecyl ammonium chloride (BDMDAC), benzalkonium chloride (BAC) and ciprofloxacin (CIP) against *P. fluorescens* and *E. coli* wild-type strains. Results show mean ±SD of three replicates**

| Strains | MIC (mg/L) | | | MBC (mg/L) | | |
|---|---|---|---|---|---|---|
| | BDMDAC | BAC | CIP | BDMDAC | BAC | CIP |
| ***E. coli*** | 6.30 | 6.30 | 0.02 | 12.5 | 25.0 | 0.09 |
| ***P. fluorescens*** | 12.50 | 12.50 | 0.05 | 50.0 | 25.0 | 0.09 |

The MIC and MBC values obtained for BDMDAC were found to be 12.5 mg/L and 50.0 mg/L for *P. fluorescens* and, 6.3 mg/L and 12.5 mg/L for *E. coli*, as showed in table 4.1. The MIC range reported for quaternary ammonium compounds (QAC's) after exposure to

gram-negative bacteria in planktonic state are often reported to be between 20 to 80 mg/L [54, 187]. The bacteria strains tested can be categorized as susceptible to BDMDAC according to CLSI M100-S22 [182], where microorganisms are reported as susceptible, resistant or non-susceptible to the drugs tested. The difference between MIC-values showed that *E. coli* is more susceptible to BDMDAC when compared with *P. fluorescens* strain. The lower MIC indicates that BDMDAC has a higher antimicrobial effect against *E. coli* strains.

As it can be verified, the inhibitory and bactericidal concentrations were all below the in-use concentrations found on products formulations with QAC's. For disinfection procedures, the concentration of biocides is 50 mg/L [54, 179]. As the MIC value was lower, it can be stated that BDMDAC still remains effective for such applications. Recently, it was reported the use of QAC's in health centers with concentrations close to 2000 mg/L, these products were considered as "low-level disinfectants" [187].

BAC and CIP MIC-values for both bacteria were also measured and were found to be 6.30 mg/L and 0.02 mg/L for *E. coli*, respectively, and for *P. fluorescens* 12.50 mg/L and 0.05 mg/L (Table 4.1). In terms of cell behavior, MIC-values observed for QAC's indicated their disinfection action on binding firmly to anionic sites present on the membrane surface, by increasing the surface pressure in the exposed membrane and decreasing the membrane fluidity [188]. These compounds as disinfectants are positively charged molecules and have a high affinity for the relatively negatively charged bacterial cells as *P. fluorescens* and *E. coli* microorganisms [54, 189, 190]. Fluoroquinolones action over gram-negative bacteria is known to be due to DNA gyrase (type I topoisomerase), this element being the most susceptible target to be inhibited by quinolones, more than topoisomerase IV (type II topoisomerase) [191, 192].

*E. coli* and *P. fluorescens* strains used in this study are considered susceptible to all antimicrobials tested for fulfilling the interpretive criteria (i.e. breakpoints) established by CLSI [182, 193, 194]. The BAC MIC-value registered was much lower than the value reported for gram-negative bacteria in another laboratory study (40 to 60 mg/L) [51]. Tabata, Nagamune [195] and Bore, Hébraud [196], showed that BAC exhibited antibacterial activity against *Pseudomonas* and *E. coli* species in concentrations between 13 to 18 mg/L, which is much closer to MIC-values observed in this work. For CIP, susceptibility typically ranges from 0.003 to 0.06 mg/L in therapeutic purposes, which agrees with the MIC value obtained for both bacterial species [192, 197, 198].

As antimicrobial susceptible tests, MICs and MBCs are used to explore the differences in bacteria susceptibility or resistance to the antimicrobial agent tested. Nonetheless, MIC values sometimes do not give an accurate information to antimicrobial susceptibility linked to the concentration that really inhibits bacteria growth [13]. Hence, the determination of MBCs is a more appropriate methodology providing the comparison of lethality between susceptible and resistant strains. In this point, the standard strains represent the population of bacteria which are

normally susceptible to the biocide. Summarizing, it defines the concentration needed to kill most cells (>99.9%) after 24h incubation under a given set of conditions [13, 16, 199].

Concerning MBC values, the most active bactericidal compound against both bacteria was CIP (with 0.09 mg/L) since it presented the lowest value of MBC. The most effective AMBs was BDMDAC (12.50 mg/L) for *E. coli*, then BAC with 25.00 mg/L for two microorganisms ending with BDMDAC against *P. fluorescens* (almost 4xMIC). This latter evidence emphasizes that BAC can be possible alternative to BDMDAC for greater disinfection.

The MIC/MBC values obtained are in the range of those reported in other studies. All of AMBs are considered as bactericides against the microorganisms tested (MBC/MIC≤4), except CIP in case of *E. coli* (MBC/MIC=4.5) [200]. Overall, the results revealed that *E. coli* is the microorganism more susceptible to the interactions with AMBs when compared to *P. fluorescens*, as suggested by Hancock [201]. *Pseudomonas* low outer membrane permeability is about 12–100 times less than that *E. coli* bacteria, which can corroborate the obtained results. CIP demonstrated to be more effective in the inactivation of planktonic cells, than the biocides, BAC and BDMDAC. The MIC- and MBC-values ranged between 0.02 to 0.09 mg/L for the antibiotic, and 6.3 to 50.0 mg/L for the biocides, respectively.

In order to determine the concentrations of antimicrobial that interfere with the planktonic growth of the model bacteria, as well as to observe the growth pattern, the bacterial growth in the presence of BDMDAC, BAC as well CIP concentrations was followed over time.

## 4.3 Adaptive resistance induction

Biocides have become an integrated part of the industrialized world, considered as valuable agents on the control of undesirable pathogens that cause damage to various products, from food to cosmetics [202], having also a valuable role in the medical area for decontamination of medical devices [13]. Therefore, huge amounts of biocides are used in several environments in medical, industrial as well as household products, where they are applied for mostly disinfection, cleaning, antisepsis and preservative purposes [203, 204].

Disinfection is considered as a crucial step in carrying out a defined, desired hygienic status on food development and processing areas [13]. Biocides usage at critical concentrations may endorse the bacteria to adapt and grow in such environment. When microorganisms are repeatedly exposed to AMB concentrations, disinfection procedure may become inefficient, which may contribute to the development of resistant mechanisms [79, 188, 205]. Disinfectants are normally used for surface disinfection and generally in concentrations higher than their MICs, but experience indicated that several products are applied in concentrations very close to MIC-values [20, 206].

Despite the widespread usage of these antimicrobial compounds, knowledge about their mode of action, especially at sub-MIC, and the microbial response to such exposure remains relatively limited [121]. Previous studies reported the appearance of high levels of *Pseudomonas* bacteria as emergent contaminant on aqueous solutions (like contact lenses, drinking water dispensers, sanitizers) despite the routine use of disinfectants [207-209]. The event has been verified for higher concentrations of QAC's, in which pathogenic *Pseudomonas* have showed resistance to BAC and, consequently, their susceptibility had decrease to others disinfectant agents [195, 210]. A similar situation was verified for *E. coli* isolates, from clinical sources, as well as others gram-negative bacteria, that exhibited higher MIC to QAC's that was correlated with the development of antibiotic resistance [211, 212]. There is a lack of knowledge regarding the accurate information on the susceptibility decrease and antibiotics resistance following bacterial adaptation to biocides. One objective of this study was to verify alterations on the bacteria susceptibility to antimicrobial agents after submission of pure-cultures bacteria to an adaptation process to BDMDAC. Then, to also find out if the bacteria adapted to BDMDAC may show susceptibility (cross-resistance) to BAC and CIP.

The induction of adaptation of *E. coli* and *P. fluorescens* to the biocide was carried out by exposing wild-type bacteria to increasing concentrations of BDMDAC. The ability of both bacteria to survive and grow at sub-MIC concentrations was verified through macroscopic analysis (figure 4.1) followed by the kinetics determination of bacterial killing and, after 24 hours of assays, confirming through MIC and MBC.

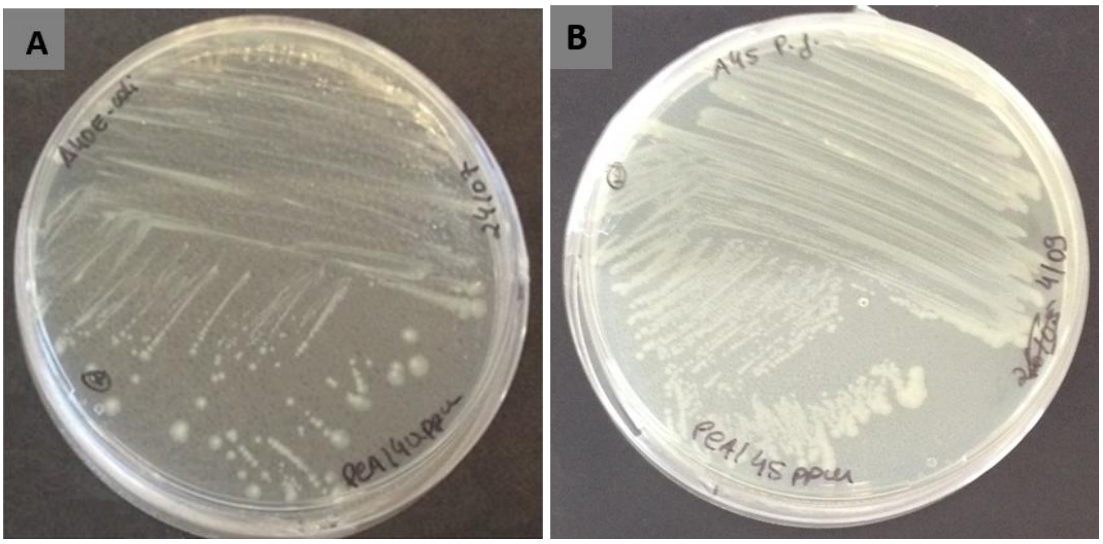



**Figure 4.1 – Bacterial cultures photographs of adapted to sub-MIC concentrations of BDMDAC: (A) *E. coli* adapted to 40 mg/L of BDMDAC and (B) *P. fluorescens* adapted to 45 mg/L of BDMDAC. Both cultures were spread into PCA supplemented with BDMDAC at the adaptation concentration.**

Figure 4.1 represents the adapted strains obtained after several passages of the bacterial cultures (wild-type) in liquid broth (TSB) supplemented with sub-MIC concentrations of

biocide. During the procedure, the growth of adapted-strains into solid medium culture was confirmed by plating the cultures in PCA supplemented with the BDMDAC at the adaptation concentration. Some differences in the culture where observed when comparing the initial pure-culture (wild-type) with the adapted strains of each bacteria. The cultures appeared to show some stress signals on the growth of bacteria spread on the PCA plate. Colonies of *P. fluorescens* and *E. coli* adapted to BDMDAC appeared to be more fluorescent than the initial culture, showing also some kind of "dry" aspect. Lamont, Beare [213] have reported the detection of a siderophore (pyoverdine) in *P. aeruginosa* that acts as a signaling molecule by triggering the expression of genes in pathogenic *Pseudomonas*, which appeared to be in the control of the production of some virulence factors that are excreted through this bacteria. In *E. coli*, the signal of fluorescence observed in the colony cell growth could be due to the presence of enterobactin, sidephores known to occur in this bacteria [214]. These observations could be pointed out as evidences on alterations of the intrinsic mechanism of bacteria resistance triggered by AMB after sub-MIC exposure to BDMDAC.

The adaptation process was achieved after the verification of an increase of the MIC and MBC values when compared with the same value obtained for the collection strains ($MIC_{WT}$; $MBC_{WT}$), as it can be observed in table 4.2.

**Table 4.2 – MICs and MBCs of BDMDAC for *E. coli* and *P. fluorescens* wild-type (WT) strains and adapted to BDMDAC**

| Strains | BDMDAC (mg/L) | |
|---|---|---|
| | MIC | MBC |
| ***E. coli* - WT** | 6.3 | 12.5 |
| **BDMDAC-adapted *E. coli*** | 25.0 | 50.0 |
| ***P. fluorescens* - WT** | 12.5 | 50.0 |
| **BDMDAC-adapted *P. fluorescens*** | 50.0 | 100.0 |

For *E. coli* the adaptation cycle was stopped after the concentration of 40 mg/L of BDMDAC ($6xMIC_{WT}$) and for *P. fluorescens* after the passage in 45 mg/L ($4x\ MIC_{WT}$) of biocide.

The MIC- and MBC-values obtained for the adapted strains have increased when compared to the MICs and MBCs recorded for the parent strains. The concentration of BDMDAC needed to completely inactivate the growth of suspended cultures was about 50 mg/L ($8xMIC_{WT}$) for adapted-resistant *E. coli* (*E. coli* A40) and for *P. fluorescens* resistant-strain (*P. fluorescens* A45; $4xMIC_{WT}$), by being adapted to 45 mg/L of BDMDAC-biocide. Concerning the adaptation procedure, *E. coli* strain adapted more quickly to BDMDAC, which contrasts with what is suggested in literature. Several authors have

demonstrated that *Pseudomonas spp* can easily adapt to sub-MIC concentrations with an underlying intrinsically resistant mechanism based on low outer-membrane permeability [116, 210]. However, it was also reported that bacterial phenotypic changes induced through the QAC's adaptation with the product dispersed in solution are frequently unstable, since when these bacteria cells are cultivated in the absence of the AMB, the resistance phenotype is quickly lost and bacteria return to wild-type phenotype [195].

### 4.3.1 Adaptation to BDMDAC in solution

When biocides are used for treatment at several environments like food production or in clinical field for general use in disinfection and cleaning, there critical areas in which bacteria are exposed to AMB residues (sub-MIC values) [215]. In order to investigate the adaptation of bacteria to BDMDAC under such conditions, repeated measurements of the MIC were done along the adaptation process. In order to confirm the changes in the MIC values, and to observe differences in the growth pattern of the strains from the culture collection and those adapted to BDMDAC, the kill curves were assessed at 25 mg/L of BDMDAC (Figure 4.2).

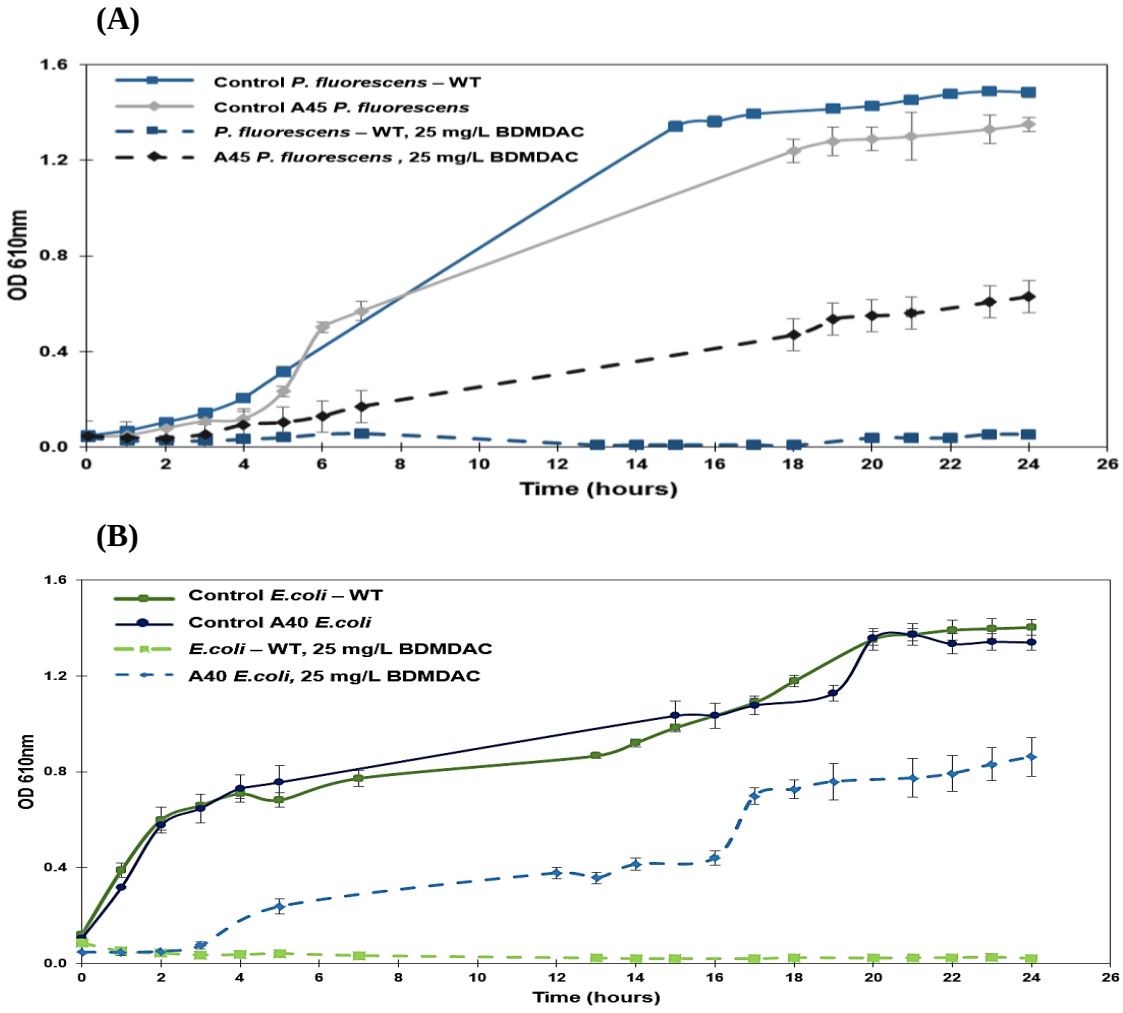



**Figure 4.2 – Time-kill curves of *P. fluorescens* (A) and *E. coli* (B) wild-type (WT) and adapted strains (A45, A40) in the presence of 25 mg/L of BDMDAC.**

Bacteria growth profiles in the absence of BDMDAC are similar, and strain independent (wild-type and adapted). Nonetheless, in the presence of 25 mg/L of BDMDAC, adapted bacteria were able to growth until an OD value of 0.6 for *P. fluorescens* and 0.8 for *E. coli*, despite showing an unusual grow pattern. In fact, the adapted bacteria growth did not follow the normal lag phase, exponential and stationary phase. Wild-type bacteria were not able to grow in presence of 25 mg/L of BDMDAC.

The MIC values of BDMDAC for *P. fluorescens* and *E. coli* prior to exposure to sub-MIC concentrations of QAC were 12.5 and 6.3 mg/L, respectively. After the adaptation process, the maximum concentration of BDMDAC that allowed bacterial growth was about 50.0 mg/L for two bacteria. With the increase in the MIC values and the growth pattern changes, it can be observed that the adapted resistant state was attained for the two bacteria.

***Stability of Adaptive resistance***

Adaptive resistance (ADR) to BDMDAC stability was analyzed by sub-culturing cells every 24 h in biocide-free TSB. The MIC was determined after each passage using the procedure described in section 3.3.1. The MIC of *P. fluorescens* A45 was stable for the first seven passages (25 mg/L), but then decreased from 25 to 12.5 mg/L BDMDAC in the last 3 passages in TSB, returning to the wildtype phenotype. The MIC of *E. coli* A40 remained stable during the 10 passages in TSB (Figure 4.3).

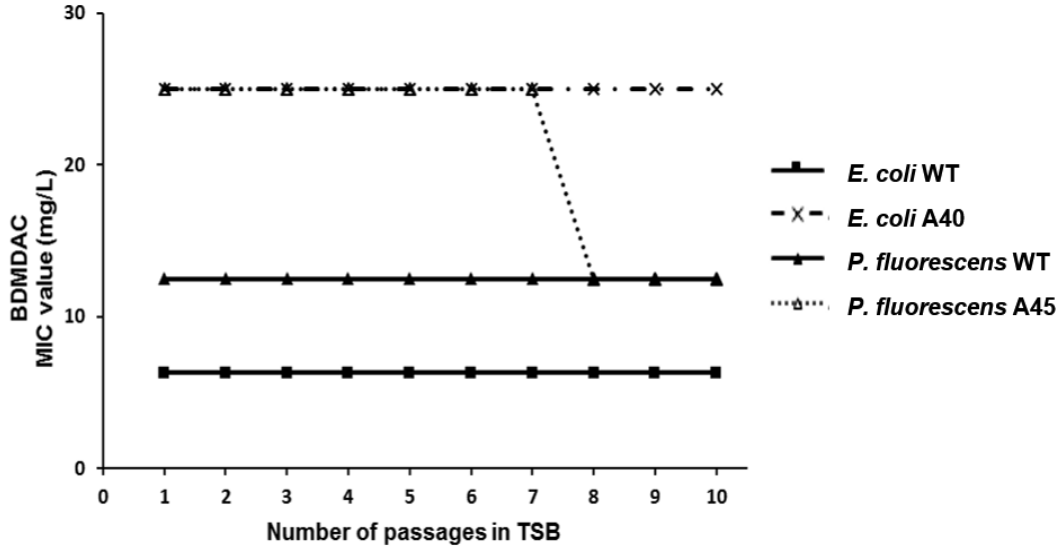



**Figure 4.3 – Stability of adaptive resistance to BDMDAC developed by *E. coli* and *P. fluorescens* strains.**

It can be assumed that the extent of developed ADR was greater in *E. coli*. Moreover, during the adaptation process *E. coli* became resistant more quickly than *P. fluorescens*. These latter facts emphasize that major concern should be taken when using biocides, since BDMDAC

(like BAC) are commonly used as domestic disinfectant products, which are often used inappropriately and at sub-inhibitory concentrations [25, 216].

The bacteria growth at biocide sub-MIC may have led to a selective pressure that induced bacteria adaptive resistance resulting in the emergence resistant clones phenotypes [8, 188, 205] and heritable mutations [217, 218] which remained after 7 successive passages in biocide-free broth. In order to understand the behavior of those strains, the kill curves of the wild-type and adapted strains are shown and discussed in the following section.

### 4.3.2 Kill curves

The microorganism response to antimicrobial agents depends on the exposure time as well as on the concentration applied. The effect of these factors on bacteria growth is not linear which might also differ between bacterial species [21, 219]. Time kill curves were used in this study to determine the kinetic pattern of bacterial death when in contact with the AMB at several concentrations over time. Results are presented in Figures 4.4 to 4.7.

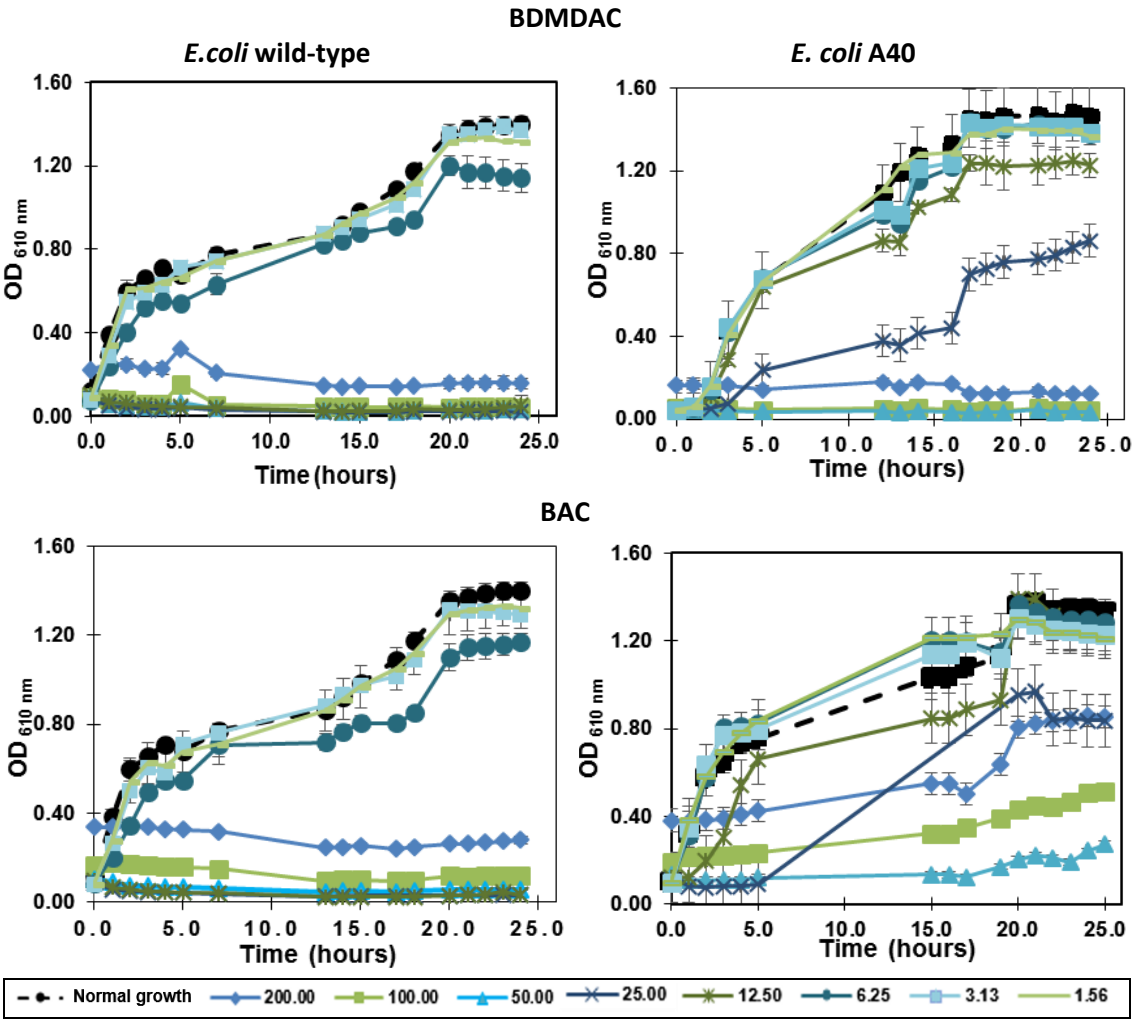



**Figure 4.4 – Time-kill curves of *E. coli* wild-type (WT) and adapted strains (A40) in the presence of increasing concentrations (mg/L) of BDMDAC and BAC. The black line represents growth in TSB. Results show mean of three replicates ±SD**

The evaluation of the growth patterns allows also to assess if bacterial killing is concentration and/or time dependent [220]. Through the analysis of growth profiles (fig. 4.4) it can be observed that the rate and extent of killing increased progressively with the increase of AMB concentrations. It can be thus stated that the observed bacterial killing profiles are concentration-dependent kill curves, as proposed by Pankey and Sabath [220].

In the absence of AMBs, the profile of bacterial growth of *E. coli* strains during 24 h was similar for adapted and non-adapted cells. Regarding *E. coli* wild-type strain growth, the exposure to 1.56 and 3.13 mg/L of BDMDAC and BAC did not have any significant influence in the microorganism growth. However, for the concentration of 6.25 mg/L of both antimicrobial agents (fig. 4.4) bacterial growth was slightly retarded since the OD values were lower than the ones obtained for normal growth, although maintaining the same pattern. The presence of 12.5 mg/L and higher concentrations of BDMDAC and BAC promoted complete inhibition of *E. coli* wild-type strain growth. This inhibition of growth due to presence of the higher biocide concentration showed that cationic surfactants were toxic for the cells leading to total inactivation right after just one hour in contact with biocides.

Concerning the *E. coli* strain adapted to BDMDAC, the analysis of the kill curves reveals that the complete growth inhibition was only verified at a BDMDAC concentration of 25 mg/L, which is also the MIC value of the adapted strain for this AMB. The complete eradication of bacterial growth was only attained for BDMDAC concentrations of 50, 100 and 200 mg/L and for BAC concentrations of 50 and 100 mg/L.

When comparing the absorbance values of the bacteria growth (wild-type and BDMDAC-adapted *E. coli*) at 25 mg/L of BDMDAC, it can be observed that the adapted bacteria was able to grow, even with some delay in the exponential phase of growth, while the same concentration completely inhibited the wild-type strain growth. This fact can corroborate the *E. coli* adaptation to BDMDAC through sub-MICs exposure. In this case, for the BDMDAC-adapted *E. coli* strain the presence of 50 mg/L of BDMDAC ($4xMBC_{WT}$) was the concentration that showed bactericidal effect.

The kill curves pattern of BAC (figure 4.4), showed that the BDMDAC-adapted strain was also able to growth in the presence of 25 mg/L of BAC. This latter fact could evidence the development of cross-resistance between BDMDAC and BAC. Comparing growth patterns of the two *E. coli* strains in the presence of BAC, it was also verified that the BDMDAC-adapted strain was able to growth in higher concentrations of BAC than the wild-type strain.

The growth pattern of the wild-type and adapted strains in the presence of an antibiotic, ciprofloxacin, was also evaluated in this study (Figure 4.5).

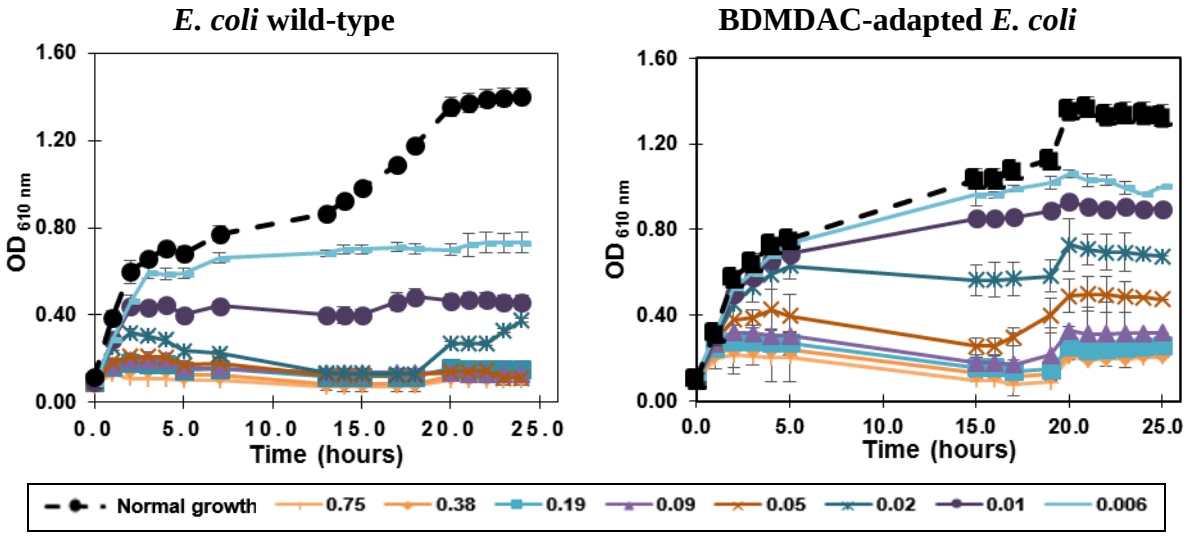



**Figure 4.5 - Time-kill curves of *E. coli* wild-type (WT) and adapted strains (A40) in the presence of increasing concentrations (mg/L) of Ciprofloxacin (CIP). The black line represents growth in TSB. Results show mean of three replicates ±SD**

Analyzing the growth profiles of wild-type strain during exposure to different concentrations of CIP, it can be observed that all the concentrations tested influence bacterial growth. In fact, even for the lower concentration tested (0.006), after 24 h, the OD values reached 0.8. For the concentration of 0.02 mg/L of CIP, it could be observed a delay in growth, the highest value of OD being observed between the 16$^{th}$ and 24$^{th}$ hours, reaching a value close to 0.4, which was the same value observed for the growth in the presence of 0.01 mg/L of CIP.

In the absence of CIP, the bacterial growth profile of *E. coli* strains during 24 h was similar for adapted and non-adapted cells. Regarding the BDMDAC-adapted *E. coli* growth in the presence of CIP, it can be observed that the extent of killing increases progressively for higher CIP concentrations. This latter fact is also a typical concentration-dependent kill curve, as proposed by Pankey and Sabath [220]. The growth pattern of the adapted strain (fig. 4.5) reveals that 0.006 and 0.01 mg/L showed OD values similar to normal growth, resulting in growth curves with similar profiles until 7 h of incubation. After 7 h, in the presence of 0.006 and 0.01 mg/L of CIP, the growth profiles showed some decrease in the OD values when compared with the curves in the absence of antibiotic. The analysis of these curves may indicate that the cross-resistance to CIP may occur after adaptation to BDMDAC. Before adaptation to BDMDAC, *E. coli* revealed to have a complete growth inhibition in presence of 0.05 mg/L of CIP, while for the adapted strain, the concentration that effectively reduced growth until 24 h was 0.09 mg/L (3x$MIC_{WT}$). Capita, Riesco-Peláez [218] reported that *E. coli* exposure to increasing sub-inhibitory concentrations of QAC's was associated with the reduction in the susceptibility to several antibiotics, including ciprofloxacin.

The same study of bacterial kill-curves profile was also performed for *P. fluorescens* (Figures 4.6 and 4.7).

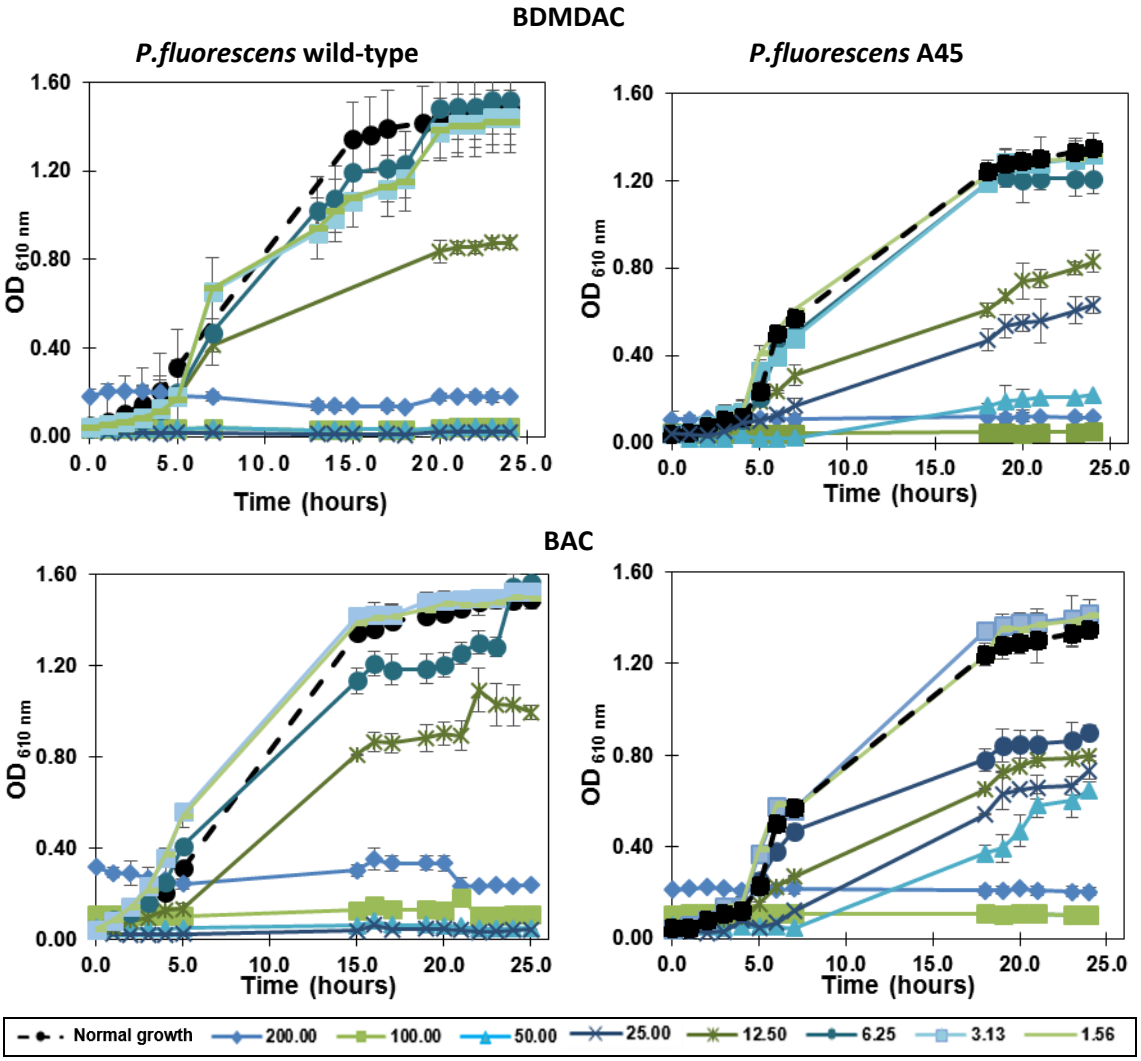



**Figure 4.6 – Time-kill curves of *P. fluorescens* wild-type (WT) and adapted strains (A45) in the presence of increasing concentrations (mg/L) of BDMDAC and BAC. The black line represents growth in TSB. Results show mean of three replicates ±SD**

In the absence of AMBs, the bacterial growth profile of *P. fluorescens* strains during 24 h was similar for adapted and non-adapted cells. Results showed that *P. fluorescens* collection strain growth (figure 4.6) in the presence of 12.50 mg/L of BDMDAC and BAC was altered being verified a slow on the bacteria growth, revealed by lower values of OD. In the case of BDMDAC kill-curves of the wild-type strain (at 12.50 mg/L), a similar trend of the growth profile obtained for growth control was also observed. For higher than 25 mg/L biocide concentrations, no *P. fluorescens* growth was observed. This complete inhibition of growth showed that for the AMB studied, under the conditions of this work, 25 mg/L was lethal for the cells, leading to total inactivation after 1h of exposure.

Concerning the BDMDAC-adapted *P. fluorescens* strain (*P. fluorescens* A45), the observation of kill curves (fig. 4.6), shows that the complete growth inhibition was only detected for a BDMDAC and BAC concentration of 50 mg/L ($MIC_{A45}$=50 mg/L).

For BDMDAC and BAC concentrations of 12.50 mg/L and higher, bacteria growth seems to have the pattern affected in a concentration-dependent manner.

Comparing the kill-curves profiles recorded for 25 mg/L and higher concentrations for both strains studied, it can be seen that the adapted bacteria was able to grow in the presence of 25 mg/L of BDMDAC and 50 mg/L of BAC (fig. 4.6). This increase in the MIC values, suggests that the *P. fluorescens* induced resistance to BDMDAC can cause the development of BAC resistance. Bacteria can thus have developed a resistance mechanism that enables the survival in contact with other antimicrobial molecules, possibly sharing the same resistance mechanism. This latter evidence suggests the possibility of emergence of cross-resistance between two biocides from the same antimicrobial class, as reported by Davin-Regli [8]. Several authors also reported that the determination of bacterial growth kinetics in the presence of low biocide concentrations may indicate changes in the bacterial phenotype [61, 121, 221]. For the complete reduction *P. fluorescens* A45 viability a increase of 8-fold $MIC_{WT}$ for BDMDAC (100 mg/L) as well BAC (100 mg/L) was needed.

Concerning the bacterial growth patterns in the presence of CIP (figure 4.7), it was observed that bacterial growth was inhibited with 0.05 mg/L and that the complete inactivation of cells viability was achieved at 0.09 mg/L for *P. fluorescens* wild-type strain.

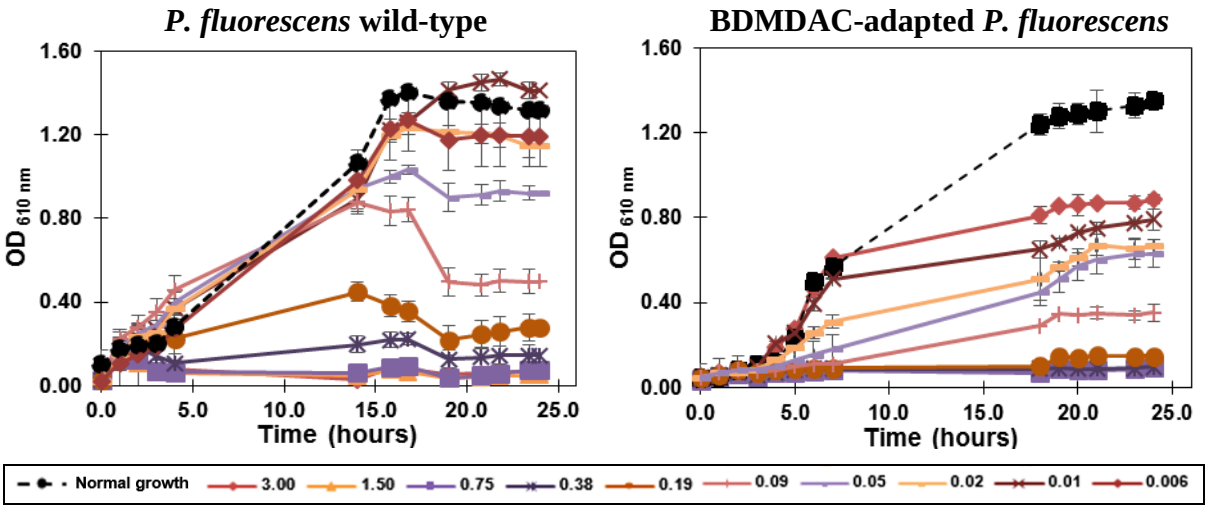



**Figure 4.7 - Time-kill curves of *P. fluorescens* wild-type (WT) and adapted strains (A45) in the presence of increasing concentrations (mg/L) of Ciprofloxacin (CIP). The black line represents growth in TSB. Results show mean of three replicates ±SD**

In the absence of CIP, the profile of bacterial growth of *P. fluorescens* strains during 24 h was similar for adapted and non-adapted cells. The presence of 0.09 mg/L of CIP affected the growth of *P. fluorescens* A45 causing some retarding on the cell growth and the complete reduction of viability of adapted-BDMDAC cells (Fig. 4.7). For the higher CIP concentrations tested (0.75, 1.5 and 3 mg/L), in both *P. fluorescens* strains, a concentration-dependent bacterial activity was observed.

At lower CIP concentrations (0.006, 0.01 and 0.02 mg/L), *P. fluorescens* had similar cell growth patterns as in the absence of CIP. However, the BDMDAC-adapted *P. fluorescens* when exposed to all CIP concentrations tested (fig.4.7), revealed reductions in the OD values for all the time-points tested. This latter fact reinforces the phenomenon that exposure to BDMDAC to sub-MIC may have led to the increase of bacteria susceptibility to CIP, since the MIC decreased in the case of the adapted strain. Results show that the previous adaptation to BDMDAC did not have any influence on the widespread selection or reduced susceptibility of *P. fluorescens* to CIP.

The MBC value was also determined and corroborated the MIC values. However, for *E. coli* A40 in contact with BAC, a clear increase of two-fold OD-value after 13 h of exposure was observed. This latter fact can be possibly justified, since BAC may have turned into micelles configuration as suggested by Lambert and Pearson [222]. In this case, there is a reduction in the BAC bactericidal activity in solution which in turn affected the emission of absorbance intensity, and consequently the OD-values.

All the kill-curves revealed to have a really short Lag-time, inferior to one hour, followed by approximately an eight hours exponential growth-phase. During this Lag-time, the growth pattern could be affected by the bacterial adaptation to the stress environment triggered by higher AMBs concentrations. As a summary, BDMDAC, BAC and CIP showed a concentration-dependent pattern of bactericidal activity.

Concerning the biocides action, their chemical nature influences the antimicrobial properties. The mechanism of action of BDMDAC and BAC, as cationic detergents, is reported to cause the disruption of the bacteria cell membrane, then the diffusion across the cell wall occurs, followed by the release and precipitation of cell components, ending on cellular lysis. In this work, results showed that BAC was more effective in the inactivation of planktonic cells then BDMDAC. In this line, BAC can be suggested as a QAC with higher alkyl-chain length than BDMDAC since it showed an higher antimicrobial action affecting the outer membrane (OM) of gram-negative bacteria in a more extensive way [223]. Additionally, it was also found that the adaptation to BDMDAC was visible through the changes of bacterial kinetics patterns. The differences observed in the kill-curves and the increase in MIC values of the adapted cells (resistant-strains) may be related with bacterial phenotype alterations [121, 224].

### 4.3.3 Cross-resistance

Recently, a relationship has been found between high QAC MICs and acquired resistance mechanism that enables microorganisms to survive in the presence of several antimicrobial molecules with similar mechanisms of action [13]. In this work, the impact of

biocide use on the reduced susceptibility of bacteria to BAC and CIP was investigated, including the case of bacteria that were previously adapted to BDMDAC (table 4.3).

**Table 4.3 – MICs and MBCs of BAC and CIP for *E. coli* and *P. fluorescens* wild-type (WT) strains and adapted to BDMDAC ($A_{40}$, $A_{45}$)**

| Strains | MIC (mg/L) | | MBC (mg/L) | |
|---|---|---|---|---|
| | BAC | CIP | BAC | CIP |
| *E. coli* WT | 6.30 | 0.02 | 25.0 | 0.05 |
| *E. coli* A40 | 25.0 | 0.05 | 50.0 | 0.09 |
| *P. fluorescens* WT | 12.50 | 0.05 | 25.0 | 0.09 |
| *P. fluorescens* A45 | 50.0 | 0.09 | 100.0 | 0.19 |

Following adaptation to BDMDAC, an increase in MIC values was observed, this being higher in *E. coli* than in *P. fluorescens*. Adaptation to BDMDAC reduced bacterial susceptibility to BAC in 4-fold for both microorganisms and 2-fold to CIP. The occurrence of cross-resistance between biocides and antibiotics in gram-negative bacteria was already reported [25, 225].

Adair, Geftic [210], Loughlin, Jones [226] and Pagedar, Singh [227] showed that *Pseudomonas* adapted to QAC had decreased susceptibility to other membrane-active agents. In a study of Carson, Larson [211] bacteria adapted to QAC's exhibited cross-resistance to dissimilar compounds, like antibiotics. However, Loughlin, Jones [226] showed that bacteria exposure to a biocide reduced its susceptibility to QAC's but did not conferred resistance to clinically relevant antibiotics. In this study, *P. fluorescens* adapted to BDMDAC bacteria showed not to have increased resistance to CIP when the growth curves were analyzed (Fig. 4.7). However, when considering only the results obtained for the MIC and MBC determination (Table 4.3), adapted *P. fluorescens* bacteria showed increased susceptibility to CIP, suggesting that adaptation may had conferred cross-resistance [86]. Although the MIC- and MBC-values registered an increase when compared to those obtained for *P. fluorescens* parent cells, the intrinsic susceptibility measured in these standard methods sometimes does not give a fixed value [228].

In *E. coli*, the reduced susceptibility to BAC and CIP was more visible through the analysis of the kill-curves and MIC/MBC determinations. Mc Cay, Ocampo-Sosa [229] in a study made with triclosan-adapted to diarreagenic *E. coli*, also verified that bacteria expressed decreased susceptibility to antibiotics and biocides. These findings revealed a high ability of *E. coli* to adapt to various stress conditions. The misuse of such compounds in food processing environments for purposes of cleaning processes, may lead to sub-lethal exposure and, consequently, to resistance to other antimicrobials [13, 205]. The mechanisms involved in the adaptive response to QAC in this study were not identified; however, those seem to be

non-specific since a reduced susceptibility was observed for both biocide (BAC) and antibiotic (CIP).

Overall, results showed that widespread selection caused by BDMDAC adaptation, reduced the susceptibility to BAC and CIP, promoting *E. coli* and *P. fluorescens* resistant strains. The bacteria showed to easily adapt to cationic surfactant – BDMDAC and the change in susceptibility revealed to be stable, in higher extent for *E. coli* CECT 434. At cell level, the adapted strains may be over-expressing efflux pumps, as mechanism of action reported for resistance to QAC, by which would further add to the burden of antimicrobial resistance already mentioned [230].

## 4.4 Antibacterial microparticules

The use of particles, at nano- or micro-scale, has being shown to be a promising strategy to prevent and treat bacterial contaminations in several fields, like surface disinfection, water treatment, food preservation, as well as drug carriers for pharmaceutical purposes [10]. This new way of delivering antibacterial agents has gained particular importance since it allows the development of new tools to overcome bacterial resistance [8, 231].

In order to evaluate the potential biological interactions and impacts of microparticle when introduced into diverse environments, it is imperative to characterize them in terms of physicochemical properties [232]. These parameters include size (surface area), configuration, surface charge, agglomeration level and antibacterial functionalization [233]. In this study BDMDAC-coated microparticles (MPs) where physically characterized and their antimicrobial action was evaluated at different concentrations against wild-type and adapted strains.

### 4.4.1 Antibacterial MPs Characterization

The bactericidal effect of MPs depends mostly on their configuration as well on the antibacterial agent applied. The characterization of the MPs surface was performed by CryoSEM (at CEMUP, Portugal), in absence of and when functionalized with BDMDAC by Figures 4.8 and 4.9.

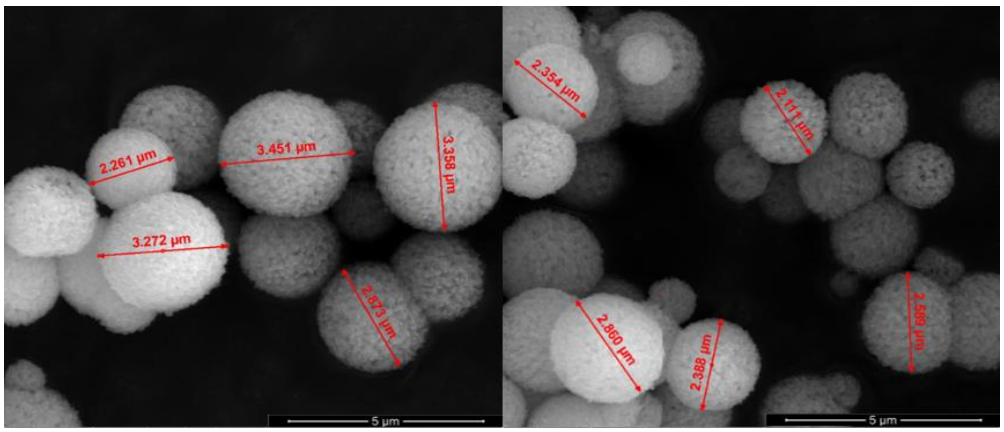



**Figure 4.8 – SEM images of $CaCO_3$ MPs size distribution. Images obtained by SEM (CEMUP). Scale bar is 5 µm for both images.**

SEM images of $CaCO_3$ MPs (Fig. 4.8) revealed a distribution of the microstructures size ranging between 2–4 µm, with an average diameter of 2.11-3.45 ± 0.49 µm. This result corroborates the ones obtained by Ferreira, Pereira [171]. MP surface was then analyzed before and after the coating process (Fig. 4.9).

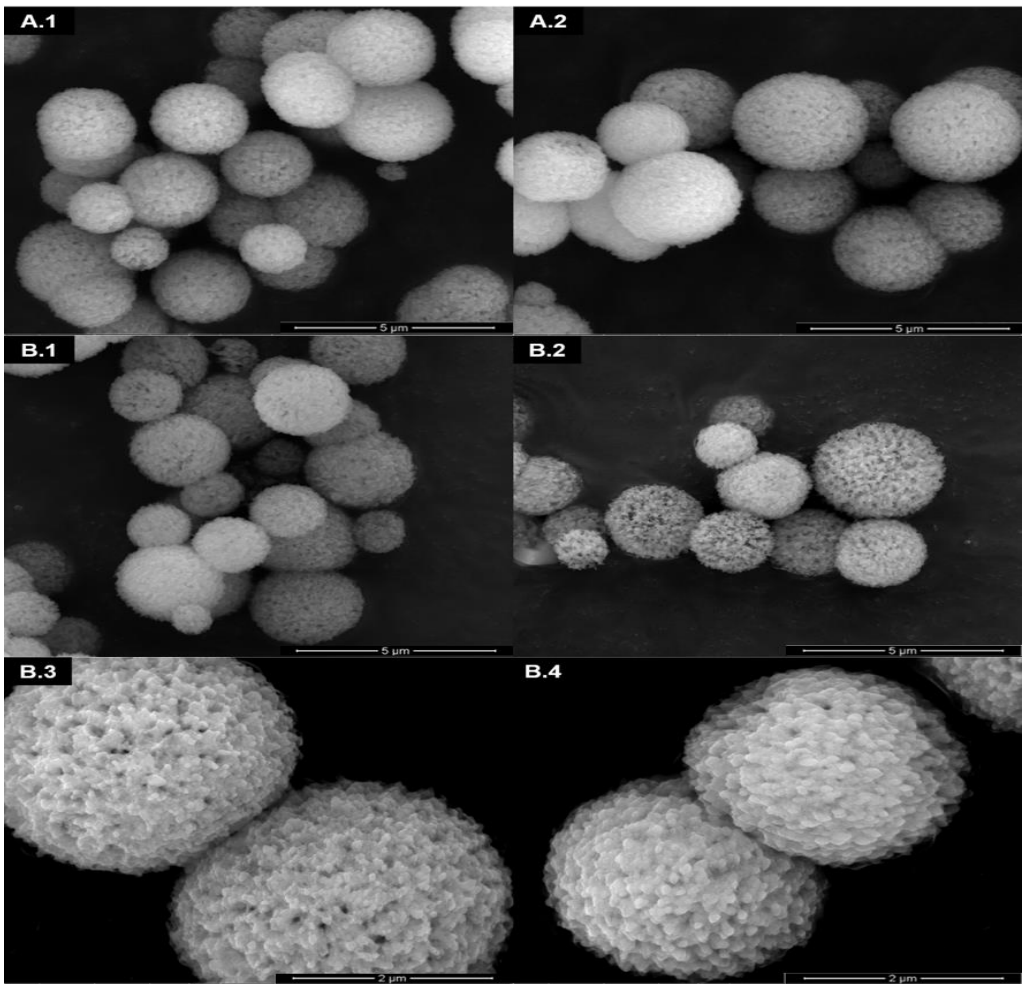



**Figure 4.9 – SEM images of MPs. Images represent the $CaCO_3$ MPs core (A.1, A.2, B.1, B.2) and MP's after coating with BDMDAC (B.3, B.4). Scale bars is 5 µm for $CaCO_3$ MPs and 2 µm for BDMDAC-$CaCO_3$ MPs.**

**Microparticles loaded with benzyldimethyldodecyl ammonium chloride to control adapted resistant bacteria**

Results showed that $CaCO_3$ and BDMDAC-$CaCO_3$ MPs had uniform spherical shape and rough surface in both cases.

After the coating process, MPs seem to have acquired a more compact core, which means that possibly some reduction of in the porosity may have occurred after functionalization. On other hand, this event could be justified by possible crystallization state of $CaCO_3$ [166]. A lower level of agglomeration was observed for uncoated MPs than for BDMDAC-coated MPs. Antimicrobial MPs surface seem to have developed channels, similar to 'reticular prolongations' (shape of a tube) which may correspond to the presence of a compound like BDMDAC that was effectively bound to MPs. Overall, this latter evidence may show that the coating process was efficient. The quantification of the BDMDAC concentration at MPs surface through the use of HPLC was not possible due to lack of resources and schedule issues. Future work will be done in order to confirm the theoretical concentration of BDMDAC adhered to the MP surface. The antimicrobial activity of BDMDAC MPs was assessed considering this theoretical value.

### 4.4.2 Microparticles antibacterial activity

The antibacterial activity of BDMDAC MPs was evaluated at different concentrations, against *P. fluorescens* and *E. coli*, by following the number of colony forming units (CFU) along time (Figures 4.10 and 4.12). The control was defined as the bacterial cultures with uncoated MPs.

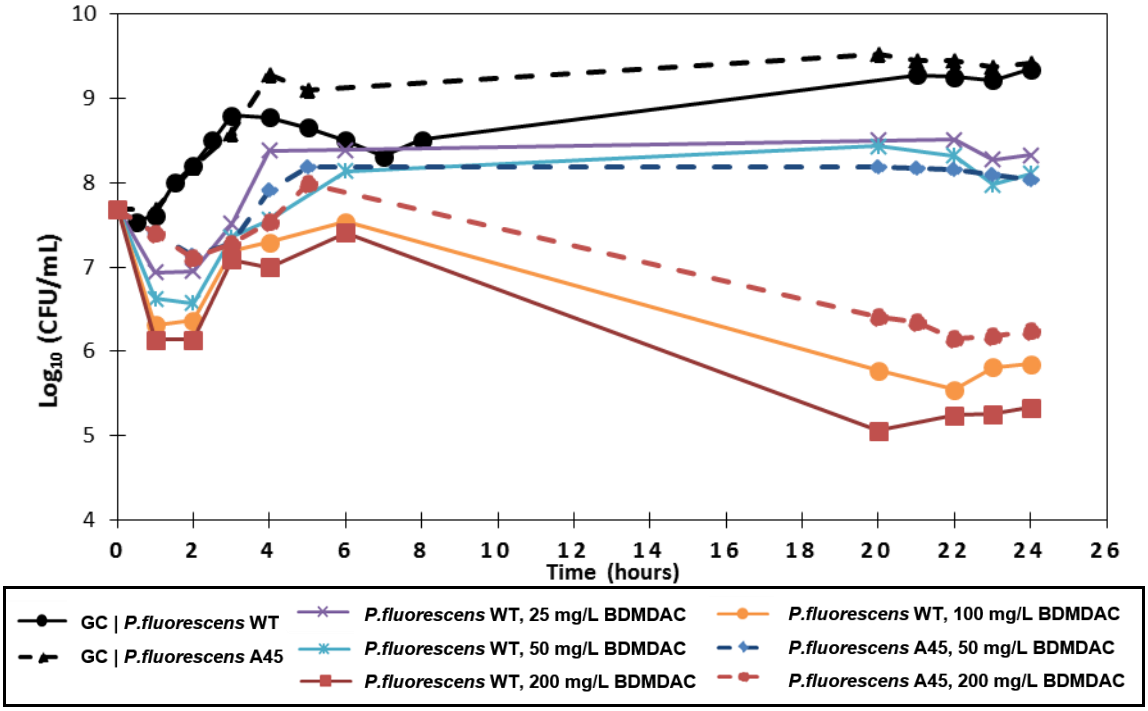



**Figure 4.10 – Kill-curves profiles of *P. fluorescens* wild-type and adapted to BDMDAC strains during exposure to several concentrations of BDMDAC MPs. CFU, colony forming units; GC, growth control.**

Results show a similar growth pattern for adapted and non-adapted cells of *P. fluorescens* strains in absence of biocide product. In general, growth (control assays) and kill curves revealed a short Lag period (two hours), followed by 3 h of exponential growth-phase. Bacteria entered than in the stationary phase being these growth curves similar to the ones obtained with the OD measurements obtained in previous section (section 4.3.2 – NG).

Concerning the growth pattern of *P. fluorescens* WT, the presence of the lower concentrations (25.0 and 50.0 mg/L) of BDMDAC MPs, after 20 h, resulted in a reduction of the number of viable cells of about 1 $\log_{10}$ cfu/mL comparing to normal growth. For BDMDAC concentrations higher than 50 mg/L a 3-log and 4-log reduction was verified in the CFU counts for 100 mg/L and, 4 for 200 mg/L respectively.

Results obtained for *P. fluorescens* A45 showed a similar behavior at the concentration of 50 mg/L of BDMDAC MPs, similar to the kill pattern of the wild-type strain. The presence of 200 mg/L induced a decline in cultivability of about 2 log when compared to the growth profile in absence of BDMDAC. When compared with the collection strain, a higher bactericidal action of 200 mg/L of BDMDAC MPs was verified against the wild-type strain (4 log reduction). In order to deeply understand the behavior of both strains after 1 h and 24 h of BDMDAC MPs exposure, a representation of these curves is shown in Figure 4.11.

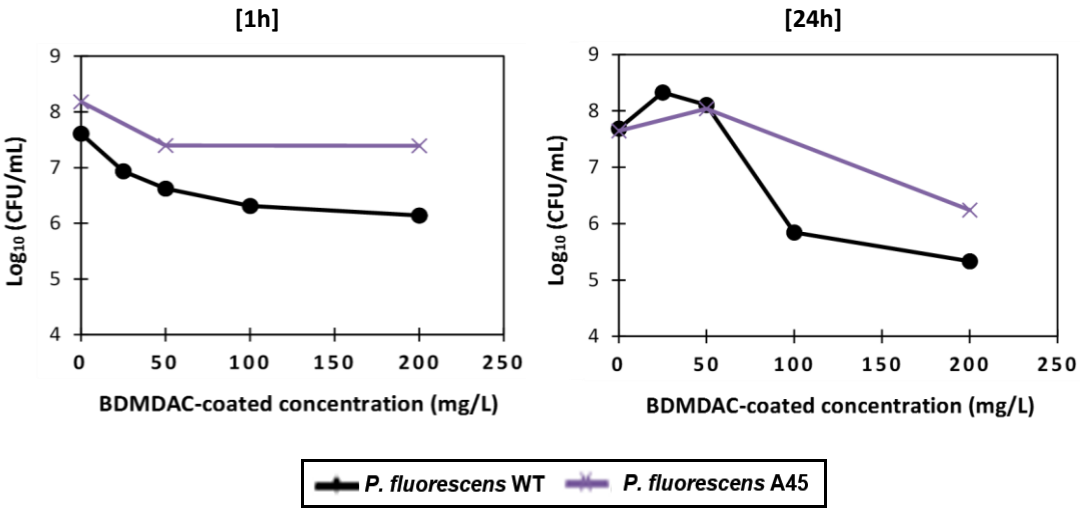



**Figure 4.11 – Bacterial cultivability (CFU) of wild-type and BDMDAC adapted *P. fluorescens*, as a function of BDMDAC-coated $CaCO_3$ MPs concentration after 1 and 24 hours of exposure.**

As it can be observed, after one hour of exposure to BDMDAC MPs, bacterial cultivability decreased with a difference of an estimated 1 $\log_{10}$ cfu/mL between the wild-type and the resistant-strain of *P. fluorescens* bacteria. After 24 h, the scenario suffered some changes on the bacterial killing profiles. The presence of BDMDAC MPs at concentrations equal or higher than 50 mg/L provided a log reduction of 1 to 2 units in the CFU counts comparing the two strains.

**Microparticles loaded with benzyldimethyldodecyl ammonium chloride to control adapted resistant bacteria**

After 1 and 24 hours in contact with BDMDAC MPs, a lower loss of *P. fluorescens* WT cultivability was verified compared with the antibacterial action caused by the BDMDAC agent dispersed in solution (Fig 4.6) during the same periods of time. Regarding the results obtained by Ferreira, Pereira [54], a lower loss in terms of bacterial cultivability in *P. fluorescens* collection strain was verified than when using the BDMDAC agent dispersed in solution.

Although this fact clearly suggests the occurrence of higher bactericidal action of BDMDAC in solution when compared with BDMDAC MPs, a special attention must be payed to the fact that the final concentration of BDMDAC on the MPs surface is a theoretical value which can be lower than the previously assumed. In this way, more tests are needed in order to define the real concentration. An identical condition could have affected the results obtained after 24 h of exposure to BDMDAC MPs (Figure 4.11).

Results obtained for the determination of BDMDAC MPs antibacterial activity against *E. coli* are shown in (Figure 4.12).

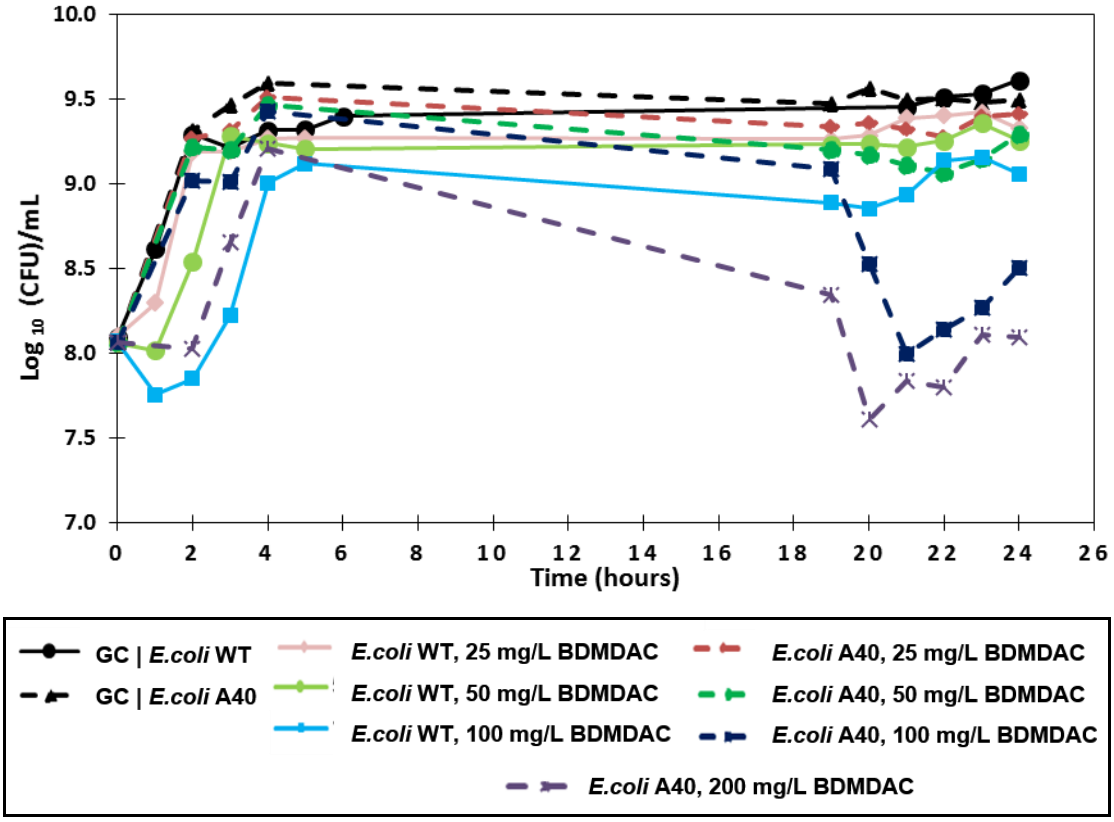



**Figure 4.12 – Kill-curves profiles of *E. coli* wild-type and adapted to BDMDAC strains during exposure to several concentrations of BDMDAC MPs. CFU, colony forming units; GC, growth control.**

The normal growth of the two *E. coli* strains was similar in pattern. Bacteria kill curves revealed a Lag period of one hour, followed by five hours of exponential growth-phase. The control reached a stationary phase, after 19 h, similarly to what happened with data collected through the OD measurements (section 4.3.2 – NG).

The *E. coli* WT growth in the presence of BDMDAC MPs, *E. coli* WT revealed a similar pattern as the normal growth despite the concentrations used.

Relatively to *E. coli* A40, it was verified, for lower the concentrations tested (25 and 50 mg/L), that the growth profiles were similar to the pattern obtained for normal growth. However, the presence of higher concentrations (100 and 200 mg/L) promoted a sharp decrease in bacterial cultivability, since a log reduction of 1-1.5 was verified, compared to the growth profiles of the control. Data were transformed in order to clearly show the BDMDAC MPs efficiency (Figure 4.13).

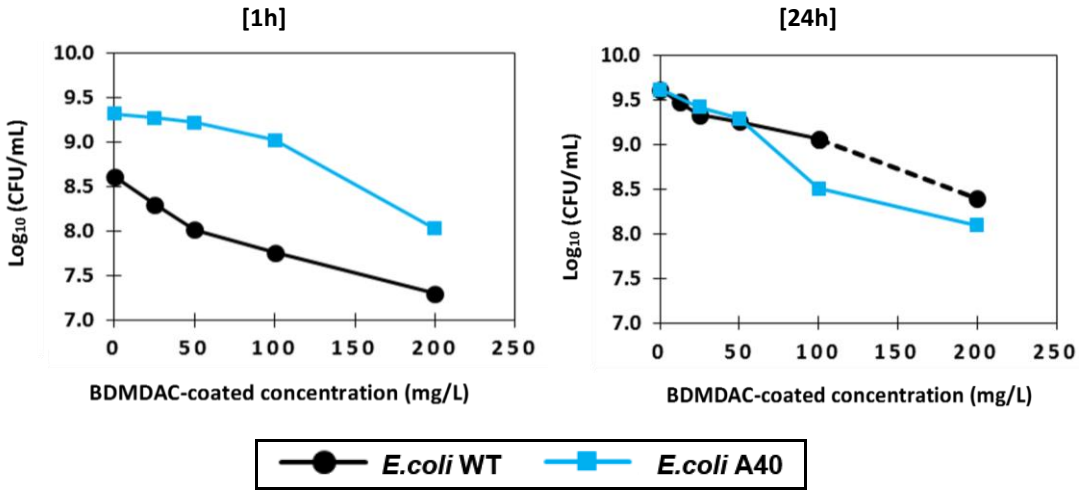



**Figure 4.13 –Bacterial cultivability (CFU) of wild-type and BDMDAC adapted *E. coli*, as a function of BDMDAC-coated $CaCO_3$ MPs concentration after 1 and 24 hours of exposure.**

As it can be observed, after one hour of exposure to BDMDAC MPs, bacterial cultivability, had declined more quickly for wild-type than for resistant-strain with a difference of log reduction correspondent to 0.5. Results obtained after 24 h show changes on the bacterial killing profiles. The presence of BDMDAC MPs concentrations equal or higher than 50 mg/L caused higher loss of cultivability of *E. coli* A40. This result may reinforce the possible use of MPs as new antibacterial agents for an alternative to commonly AMBs.

In terms of antibacterial activity, BDMDAC MPs revealed to have a higher bactericidal action against to *P. fluorescens* than to *E. coli*. This fact contrasts with what is reported in the literature, since *P. fluorescens* is considered to be more resistant than *E. coli* [201]. Comparing to the results obtained through MIC and MBC as well as with kill curves of BDMDAC in free form, the antibacterial action was greater in *E. coli* since the concentration necessary for inhibition and/or killing was lower than the one verified for *P. fluorescens* strains.

In this study, *E. coli* showed higher resistance to BDMDAC MPs when compared with *P. fluorescens* strains. On one hand, the presence of a number of small channels of porins should have conferred lower *P. fluorescens* OM permeability by helping on blocking the entrance of the BDMDAC into the bacterial cells [201]. But on the other hand, the smaller dimension of

*P. fluorescens* (rod shape, 0.5–1.0 × 1.5–5.0 μm) may partly account for the more intimate contact with the BDMDAC MPs, making the antibacterial activity more effective than for the *E. coli* (rod shape, 0.3–1.0 × 1.0–6.0 μm) [176, 209].

Regarding the antibacterial effects of BDMDAC MPs against gram negative bacteria, the bactericidal mechanism and hence the mode of action still remain to be clarified. As already mentioned, the interaction established between QAC and the cell membrane of gram-negative bacteria is through ionic and hydrophobic interactions that cause changes in the membrane properties and function [189, 230].

The model proposes that positively charged quaternary nitrogen (Figure 2.1) interacts with the head-groups of acidic phospholipids and, subsequently the hydrophobic tail integrates into the hydrophobic membrane core [189]. The antibacterial activity evidenced by BDMDAC dispersed in solution was already studied by Ferreira, Pereira [54] and confirms previous reports in the literature concerning the mode of action.

In this particular study, what remains unknown is the mechanism of action of BDMDAC when immobilized on the $CaCO_3$-MPs surface. Figure 4.14 represents a scheme as an attempt to explore the BDMDAC MPs mechanism of antibacterial action having as support the considerations of the previous findings.

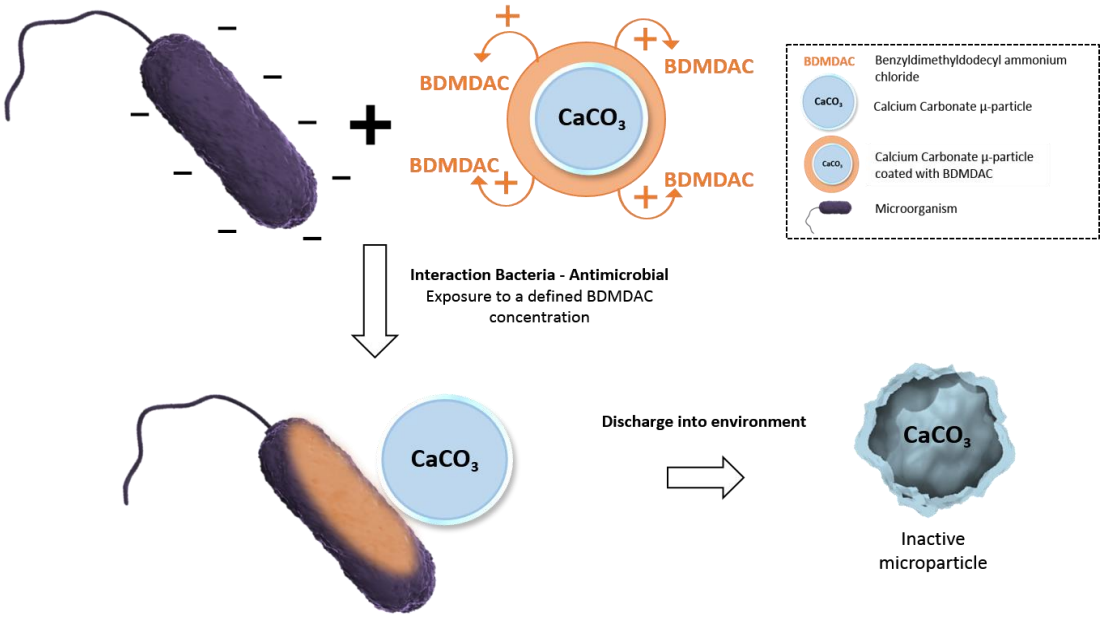



**Figure 4.14 – General mechanism of BDMDAC MPs antibacterial action - establishing the interaction between negative surface of bacteria cells and $CaCO_3$-MPs. Adapted from Moritz and Geszke-Moritz [10].**

The possible applications of AMB coated MPs would lean on the introduction in upstream of a system of water-purification membranes for example, in the design of the antibacterial treatment. Applications of such microstructures combined with membrane-systems was already reported by several authors [10, 234, 235]. In this context, a group of factors should

be taken into account, since they may influence the drug release of BDMDAC into the environment. In order to efficiently maintain microbial contaminations under control, there are several measures that should be considered: to decrease the microbial load from outside sources of the process; to efficiently control the microbial growth at vulnerable sites; to do an appropriate cleaning (concentrations defined) and disinfection of the process-lines [236].

In terms of interactions between resistant microorganisms and the components present in the wastewater, like $CaCO_3$, some concerns must be stated: i) inorganic fouling and, ii) possibly, the increase of toxicity affecting environment impact. $CaCO_3$ can be pointed out as one burden on wastewater treatment, since it may precipitate after BDMDAC release. In order to reduce their presence in wastewaters, there are several techniques that can be used to recover $CaCO_3$. Methods like flotation or implementation of the MPs into membranes system (serving as a physical barrier) have been suggested as a possible solution for $CaCO_3$ reuse [237, 238].

Since QAC's can be combined with organic compounds, their release into the environment, at low concentrations, does not affect heterotrophic bacteria [239, 240]. Other studies reported the use of QAC's like dioctadecyldimethylammonium bromide [241-245] and cetyltrimethylammonium bromide [246] to prepare antimicrobial particles that showed increased efficiency against *E. coli, S. typhimurium, P. aeruginosa, S. aureus* and *C. albicans*.

Notwithstanding, the efficiency of BDMDAC MPs depends on specific properties of MPs and bacteria. Their surface charge could be pointed out as a primary facilitator of their antimicrobial action against microorganisms. This study gives a first step in the clarification of MPs interactions with *P. fluorescens* and *E. coli*.

# Chapter V

# Concluding remarks and Research needs

Recent studies [13, 71] showed that the long-term use of antimicrobials agents in inappropriate doses in different environments for disinfection purposes increased the probability of more resistant organisms appearance. The phenomenon of bacterial resistance has been growing and efforts have been applied to find alternatives to the conventional AMBs that do not induce resistance.

The main purpose of this work was to evaluate if BDMDAC MPs could have an antimicrobial action over adapted strains of *P. fluorescens* and *E. coli*. The existence of cross-resistance of the adapted cultures to another biocide, benzalkonium chloride (BAC) and to the antibiotic ciprofloxacin (CIP), was also evaluated.

With the present work, the adaptive resistance to BDMDAC in *E. coli* and *P. fluorescens* was easily induced, as confirmed by the increase of the MIC value, as well through the changes on the growth profiles in the presence of the antimicrobial product. Moreover, the occurrence of cross-resistance to BAC and to CIP was also shown.

New microparticles of $CaCO_3$ functionalized with BDMDAC were synthetized, and their action against wild-type and adapted bacteria was assessed. The MPs showed efficient action over both bacteria strains, although not attaining 100 % efficiency of bacteria killing.

In order to efficiently determine the effective BDMDAC MPs action over the strains studied, more tests are needed, namely the determination of the BDMDAC concentration by HPLC in order to evaluate the use of MPs as new strategies of antibacterial treatments. Although the main aim of this work has been mostly achieved, there are some issues that throughout this work were raised, like the mode of action and the potential application of MPs as alternative to free BDMDAC. Additionally, it would be of interest to assess the possible

reutilization of the BDMDAC MPs developed since this is one of the main advantages referred by other studies [171].

For future work, some topics are suggested:

- To evaluate the antimicrobial potential of MPs in biofilms of wild-type and resistant strains developed in bioreactors.
- To characterize the bacterial surface of wild-type and resistant strains, in order to verify if some changes occurred after adaptation and after MPs antimicrobial action.
- To evaluate the OMP different expression through SDS-page to verify if alterations at proteins level occurred in adapted strains.
- To evaluate the risks for emerging resistance and cross-resistance in bacteria following biocide exposure through: concentration; water; soiling; exposure time; temperature; pH that might influence biocidal efficiency.
- To evaluate possible cross-resistance with others classes of AMBs and antibiotics commonly used.
- To evaluate resistance in $CaCO_3$-MPs coated with other AMB or in other cores of MPs coated with BDMDAC.
- To develop biodegradable microparticles.
- To analyze the MPs coating through XPS method for a better evaluation of the functionalized MPs.
- To perform the experimental procedure in a real domestic effluent.

# Chapter VI

# References


1. Byarugaba, D.K., *Mechanisms of antimicrobial resistance,* in *Antimicrobial Resistance in Developing Countries*2010, Springer. p. 15-26.
2. WHO, *WHO global strategy for containment of antimicrobial resistance.* 2001.
3. Davies, J. and D. Davies, *Origins and Evolution of Antibiotic Resistance.* Microbiology and Molecular Biology Reviews: MMBR, 2010. **74**(3): p. 417–433.
4. Demain, A.L. and S. Sanchez, *Microbial drug discovery: 80 years of progress.* The Journal of antibiotics, 2009. **62**(1): p. 5-16.
5. WHO, *Antimicrobial Resistance - Global Report on Surveillance.* WHO Library Cataloguing-in-Publication Data, 2014.
6. Davidson, P.M. and M.A. Harrison, *Resistance and adaptation to food antimicrobials, sanitizers, and other process controls.* Food Technology-Champaign Then Chicago, 2002. **56**(11): p. 69-78.
7. Walker, B., et al., *Looming global-scale failures and missing institutions.* Science, 2009. **325**(5946): p. 1345-1346.
8. Davin-Regli, A., *Cross-resistance between biocides and antimicrobials: an emerging question.* Revue scientifique et technique (International Office of Epizootics), 2012. **31**(1): p. 89-104.
9. Zhang, L., et al., *Development of Nanoparticles for Antimicrobial Drug Delivery.* Current Medicinal Chemistry, 2010. **17**(6): p. 585-594.
10. Moritz, M. and M. Geszke-Moritz, *The newest achievements in synthesis, immobilization and practical applications of antibacterial nanoparticles.* Chemical Engineering Journal, 2013. **228**(0): p. 596-613.
11. Chambers, H.F., et al., *Antimicrobial agents. General considerations.*, in *The pharmacological basis of therapeutics.*, N.Y.P. Press, Editor 1996: New York. p. 1026–1029.
12. Davies, J. and D. Davies, *Origins and Evolution of Antibiotic Resistance.* Microbiology and Molecular Biology Reviews : MMBR, 2010. **74**(3): p. 417-433.
13. SCENIHR, *Assessment of the Antibiotic Resistance Effects of Biocides.* Scientific Committee on Emerging and Newly Identified Health Risks, 2009.
14. EU, *98/8/EC of the European Parliament and of the Council of 16 February 1998 concerning the placing of biocidal products on the market.* Official Journal of the European Communities no. L123 of 24 April 1998, 1998.

15. EFSA, *Scientific opinion of the Panel on Biological Hazards on a request from DG SANCO on the assessment of the possible effect of the four antimicrobial treatment substances on the emergence of antimicrobial resistance.* EFSA J, 2008. **659**: p. 1-26.
16. Cerf, O., B. Carpentier, and P. Sanders, *Review: Tests for determining in-use concentrations of antibiotics and disinfectants are based on entirely different concepts: "Resistance" has different meanings.* International Journal of Food Microbiology, 2010. **136**: p. 247-254.
17. McDonnell, G. and D. Sheard, *A practical guide to decontamination in healthcare*2012: John Wiley & Sons.
18. Denyer, S.P. and G.S.A.B. Stewart, *Mechanisms of action of disinfectants.* International Biodeterioration & Biodegradation, 1998. **41**(3–4): p. 261-268.
19. Russell, A.D., *Antibiotic and biocide resistance in bacteria: comments and conclusions.* Journal of Applied Microbiology, 2002. **92**: p. 171S-173S.
20. White, D.G. and P.F. McDermott, *Biocides, drug resistance and microbial evolution.* Current Opinion in Microbiology, 2001. **4**: p. 313–317.
21. Russell, A. and G. McDonnell, *Concentration: a major factor in studying biocidal action.* Journal of Hospital Infection, 2000. **44**(1): p. 1-3.
22. Morente, E.O., et al., *Biocide tolerance in bacteria.* International Journal of Food Microbiology, 2013. **162**(1): p. 13-25.
23. Schatz, A., E. Bugle, and S.A. Waksman, *Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram-Positive and Gram-Negative Bacteria.∗†.* Experimental Biology and Medicine, 1944. **55**(1): p. 66-69.
24. Waksman, S., *History of the word 'Antibiotic'.* Journal of the history of medicine and allied sciences, 1973. **28**(3): p. 284-286.
25. Braoudaki, M. and A.C. Hilton, *Adaptive Resistance to Biocides in Salmonella enterica and Escherichia coli O157 and Cross-Resistance to Antimicrobial Agents.* Journal of Clinical Microbiology, 2004. **42**(1): p. 73-78.
26. Chandrasekaran, S. and D. Lalithakumari, *Plasmid-mediated rifampicin resistance in Pseudomonas fluorescens.* J.Med. Microbiol., 1998. **47**: p. 197-200.
27. Wright, G.D., *Bacterial resistance to antibiotics: enzymatic degradation and modification.* Advanced Drug Delivery Reviews, 2005. **57**(10): p. 1451–1470.
28. Fernández, L. and R.E.W. Hancock, *Adaptive and Mutational Resistance: Role of Porins and Efflux Pumps in Drug Resistance.* Clinical Microbiology Reviews, 2012. **25**(4): p. 661-681.
29. Ednie, L.M., M.R. Jacobs, and P.C. Appelbaum, *Comparative Activities of Clinafloxacin against Gram-Positive and -Negative Bacteria.* Antimicrobial Agents and Chemotherapy, 1998. **42**(5): p. 1269-1273.
30. Oliphant, C.M. and G.M. Green, *Quinolones: a comprehensive review.* American family physician, 2002. **65**(3): p. 455-464.
31. Brunton, L.L., *Goodman & Gilman's the pharmacological basis of therapeutics*. Vol. 12. 2011: McGraw-Hill Medical New York.
32. Hooper, D., *New uses for new and old quinolones and the challenge of resistance.* Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, 2000. **30**(2): p. 243-254.
33. Drlica, K., *Mechanism of fluoroquinolone action.* Curr Opin Microbiol, 1999. **2**(5): p. 504-508.

34. Su, H.-C., et al., *The Development of Ciprofloxacin Resistance in Pseudomonas aeruginosa Involves Multiple Response Stages and Multiple Proteins.* Antimicrobial Agents and Chemotherapy, 2010. **54**(11): p. 4626-4635.
35. Naves, P., et al., *Correlation between virulence factors and in vitro biofilm formation by Escherichia coli strains.* Microbial pathogenesis, 2008. **45**(2): p. 86-91.
36. McDonnell, G. and A.D. Russell, *Antiseptics and disinfectants: activity, action, and resistance.* Clin. Microbiol. Rev., 1999. **12**: p. 147–179.
37. Wirtanen, G., *Biofilm formation and its elimination from food processing equipment.* VTT publications 1995: p. 251pp.
38. Ferreira, C., et al., *Advances in industrial biofilm control with micro-nanotechnology.* Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology, 2010: p. 845 - 854.
39. Chapman, J.S., *Biocide resistance mechanisms.* International Biodeterioration and Biodegradation, 2003. **51**(2): p. 133-138.
40. Levy, S.B., *Antibiotic and antiseptic resistance: impact on public health.* The Pediatric infectious disease journal, 2000. **19**(10): p. S120-S122.
41. Maillard, J.Y., *Bacterial target sites for biocide action.* Journal of Applied Microbiology, 2002. **92**: p. 16S-27S.
42. Russell, A.D., *Introduction of biocides into clinical practice and the impact on antibiotic-resistant bacteria.* Journal of Applied Microbiology, 2002. **92**(Supplement s1): p. 121S–135S.
43. Gerba, C.P., *Quaternary ammonium biocides: efficacy in application.* Applied and Environmental Microbiology, 2015. **81**(2): p. 464-469.
44. McBain, A.J., et al., *Effects of quaternary-ammonium-based formulations on bacterial community dynamics and antimicrobial susceptibility.* Applied and Environmental Microbiology, 2004. **70**(6): p. 3449-3456.
45. Carmona-Ribeiro, A.M. and L.D. Melo Carrasco, *Cationic Antimicrobial Polymers and Their Assemblies.* International Journal of Molecular Sciences, 2013. **14**(5): p. 9906-9946.
46. Moore, L.E., et al., *In vitro study of the effect of cationic biocides on bacterial population dynamics and susceptibility.* . Appl. Environ. Microbiol., 2008. **74**(15): p. 4825-4834.
47. Beyth, N., et al., *Alternative Antimicrobial Approach: Nano-Antimicrobial Materials.* Evidence-Based Complementary and Alternative Medicine, 2015. **2015**.
48. Simões, M., M.O. Pereira, and M.J. Vieira, *Action of a cationic surfactant on the activity and removal of bacterial biofilms formed under different flow regimes.* Water Research, 2005. **39**(2-3): p. 478–486.
49. Merianos, J., *Surface-Active Agents.*, in *Disinfection, Sterilization, and Preservation.*, L.W. Wilkins, Editor 2001: Philadelphia, PA 19106 USA. p. 283-320.
50. Russell, A., *Bacterial adaptation and resistance to antiseptics, disinfectants and preservatives is not a new phenomenon.* Journal of Hospital Infection, 2004. **57**(2): p. 97-104.
51. Fazlara, A. and M. Ekhtelat, *The disinfectant effects of benzalkonium chloride on some important foodborne pathogens.* American-Eurasian Journal of Agricultural & Environmental Science, 2012. **12**(1): p. 23-29.
52. Mendez-Vilas, A., *Diffusivity of antimicrobial agents through biofilms of Bacillus cereus and Pseudomonas fluorescens.*, in *Worldwide Research Efforts*

*in the Fighting Against Microbial Pathogens: From Basic Research to Technological Development*, B.W. Press, Editor 2013: Florida.

53. Errer, I. and E.T. Furlong, *Identification of alkyl dimethylbenzylammonium surfactants in water samples by solid-phase extraction followed by ion trap LC/MS and LC/MS/MS.* Environmental Science & Technology, 2001. **35**(12): p. 2583-2588.
54. Ferreira, C., et al., *Physiological changes induced by the quaternary ammonium compound benzyldimethyldodecylammonium chloride on Pseudomonas fluorescens.* Journal of Antimicrobial Chemotherapy, 2011. **66**(5): p. 1036-1043.
55. Chemistry, R.S.o., *Benzyldimethyldodecyl ammonium chloride - properties.* 2015.
56. Domagk, G.A., *New class of disinfectant.* Dtsch. Med. Wochenschr., 1935. **61**: p. 829-832.
57. Vieira, D.B. and A.M. Carmona-Ribeiro, *Cationic nanoparticles for delivery of amphotericin B: preparation, characterization and activity in vitro.* Journal of nanobiotechnology, 2008. **6**(6): p. 1-13.
58. Mangalappalli-Illathu, A.K. and D.R. Korber, *Adaptive resistance and differential protein expression of Salmonella enterica serovar Enteritidis biofilms exposed to benzalkonium chloride.* Antimicrobial Agents and Chemotherapy, 2006. **50**(11): p. 3588-3596.
59. Lackner, M. and J.P. Guggenbichler, *Antimicrobial Surfaces*, in *Ullmann's Encyclopedia of Industrial Chemistry*2013, Wiley-VCH Verlag GmbH & Co. KGaA.
60. Tavlarakis, P., J. Greyling, and N.H. Snow, *Simultaneous determination of pramoxine HCl and benzalkonium chloride in wound care solutions by HPLC.* Analytical Methods, 2010. **2**(6): p. 722.
61. Gnanadhas, D.P., S.A. Marathe, and D. Chakravortty, *Biocides-resistance, cross-resistance mechanisms and assessment.* Expert opinion on investigational drugs, 2013. **22**(2): p. 191-206.
62. Ntsama-Essomba, C., et al., *Resistance of Escherichia coli growing as biofilms to disinfectants.* Veterinary research, 1997. **28**(4): p. 353-363.
63. Langsrud, S., G. Sundheim, and R. Borgmann-Strahsen, *Intrinsic and acquired resistance to quaternary ammonium compounds in food-related Pseudomonas spp.* Journal of Applied Microbiology, 2003. **95**(4): p. 874-882.
64. Denyer, S.P., *Mechanisms of action of antibacterial biocides.* International Biodeterioration & Biodegradation, 1995. **36**(3): p. 227-245.
65. Poole, K., *Mechanisms of bacterial biocide and antibiotic resistance.* Journal of Applied Microbiology, 2002. **92**(s1): p. 55S-64S.
66. van der Horst, M.A., et al., *De novo acquisition of resistance to three antibiotics by Escherichia coli.* Microbial drug resistance, 2011. **17**(2): p. 141-147.
67. Jayaraman, P., et al., *Activity and interactions of antibiotic and phytochemical combinations against Pseudomonas aeruginosa in vitro.* International Journal of Biological Sciences, 2010. **6** (6): p. 556-568.
68. Maillard, J.-Y., et al., *Does microbicide use in consumer products promote antimicrobial resistance? A critical review and recommendations for a cohesive approach to risk assessment.* Microbial drug resistance, 2013. **19**(5): p. 344-354.
69. Beumer, R., et al., *Microbial resistance and biocides.* International Scientific Forum on HomeHygiene, 2000: p. 42.
70. Andrews, J.M., *Determination of minimum inhibitory concentrations.* J. Antimicrob. Chemother., 2001. **48**(1): p. 5-16.

71. Gaude, G., *Preventing bacterial infections in the society: Lessons to be learnt*. Vol. 7. 2014. 1-5.
72. Patel, T. and A. Levitin, *Escherichia Coli Adaptive Resistance to Clinical Antibiotics.* JSM Microbiology, 2014. **2**(1: 1007): p. 1-5.
73. Tenover, F.C., *Mechanisms of Antimicrobial Resistance in Bacteria.* The American Journal of Medicine, 2006. **119**(6, Supplement 1): p. S3-S10.
74. Wise, R., *Antimicrobial resistance: increasing concerns.* The British Journal of General Practice, 2007. **57**(543): p. 772–774.
75. Lawrence, R. and E. Jeyakumar, *Antimicrobial Resistance: A Cause for Global Concern.* BMC Proceedings, 2013. **7**(3): p. 14.
76. Thabit, A.K., J.L. Crandon, and D.P. Nicolau, *Review: Antimicrobial resistance: Impact on clinical and economic outcomes and the need for new antimicrobials.* Expert Opinion on Pharmacotherapy, 2015. **16**(2): p. 159-177.
77. Bassetti, M., F. Ginocchio, and M. Mikulska, *New treatment options against gram-negative organisms.* Critical Care, 2011. **15**(2): p. 215-215.
78. SCENIHR, *Research strategy to address the knowledge gaps on the antimicrobial resistance effects*
*of biocides.* Scientific Committee on Emerging and Newly Identified Health Risks, 2010.
79. Fernandez, L., E.B. Breidenstein, and R.E. Hancock, *Creeping baselines and adaptive resistance to antibiotics.* Drug Resist Updat, 2011. **14**(1): p. 1-21.
80. Schweizer, H.P., *Mechanisms of antibiotic resistance in Burkholderia pseudomallei: implications for treatment of melioidosis.* Future microbiology, 2012. **7**(12): p. 1389-1399.
81. Park, A.J., M.D. Surette, and C.M. Khursigara, *Antimicrobial targets localize to the extracellular vesicle-associated proteome of Pseudomonas aeruginosa grown in a biofilm.* Frontiers in Microbiology, 2014. **5**: p. 1-12.
82. Gebel, J., et al., *The role of surface disinfection in infection prevention.* GMS hygiene and infection control, 2013. **8**(1): p. Doc10.
83. van Hoek, A.H.A.M., et al., *Acquired Antibiotic Resistance Genes: An Overview.* Frontiers in Microbiology, 2011. **2**: p. 203.
84. DeRyke, C.A., et al., *Optimising dosing strategies of antibacterials utilising pharmacodynamic principles: impact on the development of resistance.* Drugs, 2006. **66**: p. 1-14.
85. Olofsson, S.K. and O. Cars, *Optimizing Drug Exposure to Minimize Selection of Antibiotic Resistance.* Clinical Infectious Diseases, 2007. **45**(2): p. S129-S136.
86. Keen, P.L. and M.H. Montforts, *Antimicrobial resistance in the environment*2011: John Wiley & Sons.
87. Martínez, J.L. and F. Rojo, *Metabolic regulation of antibiotic resistance.* FEMS Microbiology Reviews, 2011. **35**(5): p. 768–789.
88. Cox, G. and G.D. Wright, *Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions.* International Journal of Medical Microbiology, 2013. **303**(6–7): p. 287-292.
89. Blair, J.M.A., et al., *Molecular mechanisms of antibiotic resistance.* Nat Rev Micro, 2015. **13**(1): p. 42-51.
90. Poole, K., *Efflux-mediated antimicrobial resistance.* Journal of Antimicrobial Chemotherapy, 2005. **56**(1): p. 20-51.
91. Costerton, J.W., P.S. Stewart, and E.P. Greenberg, *Bacterial biofilms: a common cause of persistent infections.* Science, 1999. **284**: p. 1318-1322.

92. Paraje, M.G., *Antimicrobial resistance in biofilms.* Science against microbial pathogens: communicating current research and technological advances, 2011: p. 736-744.
93. Bridier, A., et al., *Resistance of bacterial biofilms to disinfectants: a review.* Biofouling, 2011. **27**(9): p. 1017-1032.
94. Barah, F., *Non-Antibiotic Biocides: An Updated Review.* Microbial Pathogens and Strategies for Combating them: Science, Technology and Education (A. Méndez-Vilas, Ed.) Badajoz, Spain, Formatex Research Centre, 2013: p. 598-607.
95. Kümmerle, N., H.-H. Feucht, and P.-M. Kaulfers, *Plasmid-mediated formaldehyde resistance in Escherichia coli: characterization of resistance gene.* Antimicrobial Agents and Chemotherapy, 1996. **40**(10): p. 2276-2279.
96. Cloete, T.E., *Resistance mechanisms of bacteria to antimicrobial compounds.* International Biodeterioration & Biodegradation, 2003. **51**(4): p. 277-282.
97. Dzidic, S. and V. Bedekovic, *Horizontal gene transfer-emerging multidrug resistance in hospital bacteria.* Acta Pharmacologica Sinica, 2003. **24**(6): p. 519-526
98. Vogan, A.A. and P.G. Higgs, *The advantages and disadvantages of horizontal gene transfer and the emergence of the first species.* Biology Direct, 2011. **6**(1): p. 1-14.
99. Boerlin, P. and D.G. White, *Antimicrobial Resistance and Its Epidemiology*, in *Antimicrobial Therapy in Veterinary Medicine*2013, John Wiley & Sons, Inc. p. 21-40.
100. Martinez, J. and F. Baquero, *Mutation frequencies and antibiotic resistance.* Antimicrobial Agents and Chemotherapy, 2000. **44**(7): p. 1771-1777.
101. Dean, A.C.R., *Adaptive drug resistance in Gram-negative bacteria.* Proc R Soc Med, 1971. **64**: p. 534–537.
102. Skiada, A., et al., *Adaptive resistance to cationic compounds in Pseudomonas aeruginosa.* Int J Antimicrob Agents, 2011. **37**(3): p. 187-93.
103. Lee, K., et al., *Short-term Adaptive Resistance in E. coli K-12 is not dependent on acrD, acrA and tolC.* Journal of Experimental Microbiology and Immunology, 2013. **17**: p. 8 –13.
104. Bryan, L.E. and S. Kwan, *Mechanisms of aminoglycoside resistance of anaerobic bacteria and facultative bacteria grown anaerobically.* Journal of Antimicrobial Chemotherapy, 1981. **8**(suppl D): p. 1-8.
105. Karlowsky, J.A., et al., *Altered denA and anr gene expression in aminoglycoside adaptive resistance in Pseudomonas aeruginosa.* Journal of Antimicrobial Chemotherapy, 1997. **40**(3): p. 371-376.
106. Conrad, R.S., R.G. Wulf, and D.L. Clay, *Effects of Carbon Sources on Antibiotic Resistance in Pseudomonas aeruginosa.* Antimicrobial Agents and Chemotherapy, 1979. **15**(1): p. 59-66.
107. Nicas, T.I. and R.E. Hancock, *Outer membrane protein H1 of Pseudomonas aeruginosa: involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B, and gentamicin.* Journal of Bacteriology, 1980. **143**(2): p. 872-878.
108. Nicas, T.I. and R.E.W. Hancock, *Alteration Of Susceptibility To Edta, Polymyxin B And Gentamicin In Pseudomonas Aeruginosa By Divalent Cation Regulation Of Outer Membrane Protein H1.* Journal of General Microbiology, 1983. **129**(2): p. 509-517.

109. Gould, I.M., et al., *Ionic binding, adaptive resistance and post-antibiotic effect of netilmicin and ciprofloxacin.* Journal of Antimicrobial Chemotherapy, 1991. **27**(6): p. 741-748.
110. Damper, P.D. and W. Epstein, *Role of the membrane potential in bacterial resistance to aminoglycoside antibiotics.* Antimicrobial Agents and Chemotherapy, 1981. **20**(6): p. 803-808.
111. Xiong, Y.Q., et al., *Influence of pH on adaptive resistance of Pseudomonas aeruginosa to aminoglycosides and their postantibiotic effects.* Antimicrobial Agents and Chemotherapy, 1996. **40**(1): p. 35-39.
112. Samartzidou, H. and A.H. Delcour, *Excretion of Endogenous Cadaverine Leads to a Decrease in Porin-Mediated Outer Membrane Permeability.* Journal of Bacteriology, 1999. **181**(3): p. 791-798.
113. Tkachenko, A.G. and L.Y. Nesterova, *Polyamines as Modulators of Gene Expression under Oxidative Stress in Escherichia coli.* Biochemistry (Moscow), 2003. **68**(8): p. 850-856.
114. Kwon, D.H. and C.-D. Lu, *Polyamines Induce Resistance to Cationic Peptide, Aminoglycoside, and Quinolone Antibiotics in Pseudomonas aeruginosa PAO1*. Antimicrobial Agents and Chemotherapy, 2006. **50**(5): p. 1615-1622.
115. Verstraeten, N., et al., *Living on a surface: swarming and biofilm formation. .* Trends Microbiol., 2008. **16**: p. 496-506.
116. Breidenstein, E.B.M., C. de la Fuente-Núñez, and R.E.W. Hancock, *Pseudomonas aeruginosa: all roads lead to resistance.* Trends in Microbiology, 2011. **19**(8): p. 419-426.
117. Yuan, W., et al., *Cell wall thickening is associated with adaptive resistance to amikacin in methicillin-resistant Staphylococcus aureus clinical isolates.* Journal of Antimicrobial Chemotherapy, 2013. **68**(5): p. 1089-1096.
118. Machado, I., et al., *Antimicrobial Pressure of Ciprofloxacin and Gentamicin on Biofilm Development by an Endoscope-Isolated Pseudomonas aeruginosa.* ISRN Biotechnology, 2013. **2013**: p. 10.
119. Seier-Petersen, M.A., et al., *Development of bacterial resistance to biocides and antimicrobial agents as a consequence of biocide usage*, 2013, Technical University of DenmarkDanmarks Tekniske Universitet, Department of MicrobiologyInstitut for Mikrobiologi.
120. Russell, A.D., *Bacterial resistance to disinfectants: present knowledge and future problems.* Journal of Hospital Infection, 1999. **43, Supplement 1**(0): p. S57-S68.
121. Maillard, J.Y., *Bacterial resistance to biocides in the healthcare environment: should it be of genuine concern?* Journal of Hospital Infection, 2007. **65**: p. 60-72.
122. Chapman, J.S., *Disinfectant resistance mechanisms, cross-resistance, and co-resistance.* International Biodeterioration & Biodegradation, 2003. **51**(4): p. 271-276.
123. Oliveira, H., et al., *Unexploited opportunities for phage therapy.* Frontiers in pharmacology, 2015. **6**.
124. Borges, A., M. J Saavedra, and M. Simoes, *Insights on Antimicrobial Resistance, Biofilms and the Use of Phytochemicals as New Antimicrobial Agents.* Current Medicinal Chemistry, 2015. **22**(21): p. 2590-2614.
125. Xue, Y., H. Xiao, and Y. Zhang, *Antimicrobial Polymeric Materials with Quaternary Ammonium and Phosphonium Salts.* International Journal of Molecular Sciences, 2015. **16**(2): p. 3626-3655.

126. Narayana, J.L. and J.-Y. Chen, *Antimicrobial peptides: possible anti-infective agents.* Peptides, 2015. **72**: p. 88-94.
127. Weir, E., et al., *The use of nanoparticles in anti-microbial materials and their characterization.* Analyst, 2008. **133**(7): p. 835-845.
128. Campos, E., et al., *Designing polymeric microparticles for biomedical and industrial applications.* European Polymer Journal, 2013. **49**(8): p. 2005-2021.
129. Huh, A.J. and Y.J. Kwon, *"Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era.* Journal of Controlled Release, 2011. **156**(2): p. 128-145.
130. Theron, J., J. Walker, and T. Cloete, *Nanotechnology and water treatment: applications and emerging opportunities.* Critical Reviews in Microbiology, 2008. **34**(1): p. 43-69.
131. Wang, L.-S., A. Gupta, and V.M. Rotello, *Nanomaterials for the Treatment of Bacterial Biofilms.* ACS Infectious Diseases, 2015.
132. Coelho, J.F., et al., *Drug delivery systems: Advanced technologies potentially applicable in personalized treatments.* The EPMA Journal, 2010. **1**(1): p. 164-209.
133. Yadav, K.S.A., A., L.S. Vasudha, and H.G. Shivakumar, *Different techniques for preparation of polymeric nanoparticles - A Review.* Asian J Pharm Clin Res, Vol 2012. **5**(3): p. 16-23.
134. Mout, R., et al., *Surface functionalization of nanoparticles for nanomedicine.* Chemical Society reviews, 2012. **41**(7): p. 2539-2544.
135. Madhav, N.V.S. and S. Kala, *Review on Microparticulate Drug Delivery System.* International Journal of PharmTech Research, 2011. **3**(3): p. 1242-1254.
136. Hansen, S.F., A. Baun, and C. Ganzleben, *Nanomaterials and the European Water Framework Directive.* European Journal of Law and Technology; Vol 2, No 3 (2011), 2011.
137. Colvin, V.L., *The potential environmental impact of engineered nanomaterials.* Nature biotechnology, 2003. **21**(10): p. 1166-1170.
138. Cloete, T.E., *Nanotechnology in water treatment applications*2010: Horizon Scientific Press.
139. Luoma, S.N., *Silver Nanotechnologies and the Environment.* The Project on Emerging Nanotechnologies Report, 2008. **15**.
140. EPA, *Senior Policy Council. Nanotechnology White Paper.* U.S. Environmental Protection Agency, 2007.
141. Lubick, N., *Risks of nanotechnology remain uncertain.* Environmental Science & Technology, 2008. **42**(6): p. 1821-1824.
142. Morton, S., Z. Poon, and P. Hammond, *The Architecture and Biological Performance of Drug-Loaded LbL Nanoparticles.* Biomaterials, 2013. **34**(21): p. 5328-5335.
143. Decher, G., *Fuzzy nanoassemblies: toward layered polymeric multicomposites.* Science, 1997. **277**(5330): p. 1232-1237.
144. Yan, Y., M. Björnmalm, and F. Caruso, *Assembly of layer-by-layer particles and their interactions with biological systems.* Chemistry of Materials, 2013. **26**(1): p. 452-460.
145. Donath, E., et al., *Novel Hollow Polymer Shells by Colloid-Templated Assembly of Polyelectrolytes.* Angewandte Chemie International Edition, 1998. **37**(16): p. 2201-2205.
146. Ariga, K. and J.P. Hill, *Layer-by-Layer (LbL) Assembly, A "Gentle Yet Flexible" Method Toward Functional Biomaterials.* Material Matters, 2008. **3.3**( 57).

147. Caruso, F., *Generation of Complex Colloids by Polyelectrolyte-Assisted Electrostatic Self-Assembly*. Current Chemistry, 2001. **54**: p. 349-353.
148. Wang, C., et al., *Facile fabrication of hybrid colloidosomes with alginate gel cores and shells of porous CaCO3 microparticles.* ChemPhysChem, 2007. **8**(8): p. 1157-1160.
149. Won, Y.-H., et al., *Multifunctional calcium carbonate microparticles: Synthesis and biological applications.* Journal of Materials Chemistry, 2010. **20**(36): p. 7728-7733.
150. Sato, K., M. Seno, and J.-I. Anzai, *Release of Insulin from Calcium Carbonate Microspheres with and without Layer-by-Layer Thin Coatings.* Polymers, 2014. **6**(8): p. 2157-2165.
151. Lipovetskaya, Y., *Microspheres: technologies and global markets.* Wellesley: BCC Research, 2010.
152. Bansal, S.S., A. Joshi, and A.K. Bansal, *New dosage formulations for targeted delivery of cyclo-oxygenase-2 inhibitors.* Drugs & aging, 2007. **24**(6): p. 441-451.
153. Roos, M.A., et al., *Microparticles in physiological and in pathological conditions.* Cell biochemistry and function, 2010. **28**(7): p. 539-548.
154. Sørensen, G., et al., *Controlled release of biocide from silica microparticles in wood paint.* Progress in Organic Coatings, 2010. **68**(4): p. 299-306.
155. Bile, J., et al., *Antimicrobial films containing microparticles for the enhancement of long-term sustained release.* Drug development and industrial pharmacy, 2015: p. 1-7.
156. Liu, Y. and H.-I. Kim, *Characterization and antibacterial properties of genipin-crosslinked chitosan/poly (ethylene glycol)/ZnO/Ag nanocomposites.* Carbohydrate polymers, 2012. **89**(1): p. 111-116.
157. Kokura, S., et al., *Silver nanoparticles as a safe preservative for use in cosmetics.* Nanomedicine: Nanotechnology, Biology and Medicine, 2010. **6**(4): p. 570-574.
158. Kejdušová, M., et al., *Antimicrobial Properties of Microparticles Based on Carmellose Cross-Linked by Cu< sup> 2.* BioMed Research International, 2015. **2015**.
159. Pant, H.R., et al., *Photocatalytic and antibacterial properties of a TiO2/nylon-6 electrospun nanocomposite mat containing silver nanoparticles.* J. Hazard. Mater., 2011. **189**: p. 465-471.
160. Sophee, S., et al., *Antibacterial activity of TiO2 and ZnO microparticles combination on water polluting bacteria.* Journal of Green Science and Technology, 2013. **1**(1): p. 20-26.
161. Turalija, M., et al., *Copper (i) oxide microparticles–synthesis and antimicrobial finishing of textiles.* Journal of Materials Chemistry B, 2015. **3**(28): p. 5886-5892.
162. Wen, J. and A.B. Anderson, *Microparticle containing matrices for drug delivery*, 2014, Google Patents.
163. Li, W.-R., et al., *Antibacterial effect of silver nanoparticles on Staphylococcus aureus.* Biometals, 2011. **24**(1): p. 135-141.
164. Pant, H.R., et al., *Photocatalytic and antibacterial properties of a TiO 2/nylon-6 electrospun nanocomposite mat containing silver nanoparticles.* Journal of hazardous materials, 2011. **189**(1): p. 465-471.
165. Barani, H., et al., *In situ synthesis of nano silver/lecithin on wool: enhancing nanoparticles diffusion.* Colloid Surf., 2012. **B**(92 ): p. 9-15.

166. Sukhorukov, G.B., et al., *Porous calcium carbonate microparticles as templates for encapsulation of bioactive compounds.* Journal of Materials Chemistry, 2004. **14**(14): p. 2073-2081.
167. Gao, P., et al., *Recent advances in materials for extended-release antibiotic delivery system.* The Journal of antibiotics, 2011. **64**(9): p. 625-634.
168. Bruschi, M., et al., *Preparation and Antimicrobial Activityof Gelatin Microparticles Containing Propolis Against Oral Pathogens.* Drug development and industrial pharmacy, 2006. **32**(2): p. 229-238.
169. Blin, T., et al., *Bactericidal microparticles decorated by an antimicrobial peptide for the easy disinfection of sensitive aqueous solutions.* Biomacromolecules, 2011. **12**(4): p. 1259-1264.
170. Dhanalakshmi, T. and S. Rajendran, *Antimicrobial Activity of Micro Sized Copper Particles On Water Borne Bacterial Pathogens* International Journal of Scientific & Technology Research, 2013. **2**(1): p. 115-118.
171. Ferreira, C., et al., *Biofilm Control With New Microparticles With Immobilized Biocide.* Heat Transfer Engineering, 2013. **34**(8-9): p. 712-718.
172. Preisig, D., et al., *Drug loading into porous calcium carbonate microparticles by solvent evaporation.* European Journal of Pharmaceutics and Biopharmaceutics, 2014. **87**(3): p. 548-558.
173. Pecarski, D., et al., *Preparation, characterization and antimicrobial activity of chitosan microparticles with thyme essential oil.* Hemijska industrija, 2014(00): p. 48-48.
174. Jeon, S.J., et al., *Underlying mechanism of antimicrobial activity of chitosan microparticles and implications for the treatment of infectious diseases.* PloS one, 2014. **9**(3): p. e92723.
175. Khiralla, G.M. and B.A. El-Deeb, *Antimicrobial and antibiofilm effects of selenium nanoparticles on some foodborne pathogens.* LWT-Food Science and Technology, 2015.
176. Li, H., et al., *Enhancing the antimicrobial activity of natural extraction using the synthetic ultrasmall metal nanoparticles.* Scientific reports, 2015. **5**.
177. Palleroni, N.J., *Genus I. Pseudomonas Migula 1984, 237AL.* Bergey's Manual of Systematic Bacteriology, Krieg, N. R., and Holt, J. G. eds., (Baltimore: Williams & Wilkins) 1984: p. 141–199.
178. Picot, L., et al., *Pseudomonas fluorescens as a potential pathogen: adherence to nerve cells.* Microbes and Infection, 2001. **3**(12): p. 985-995.
179. Simões, M., et al., *Antimicrobial mechanisms of ortho-phthalaldehyde action.* Journal of Basic Microbiology, 2007. **47**: p. 230–242
180. Vaz-Moreira, I., O.C. Nunes, and C.M. Manaia, *Diversity and antibiotic resistance in Pseudomonas spp. from drinking water.* Science of The Total Environment, 2012. **426**(0): p. 366-374.
181. Allocati, N., et al., *Escherichia coli in Europe: An Overview.* International Journal of Environmental Research and Public Health, 2013. **10**(12): p. 6235-6254.
182. CLSI, *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-Second Informational Supplement.*, in *CLSI document M100-S22*, C.a.L.S. Institute, Editor 2012: Wayne, PA.
183. Machado, I., et al., *Proteomic approach to Pseudomonas aeruginosa adaptive resistance to benzalkonium chloride.* Journal of proteomics, 2013. **89**: p. 273-279.

184. Ferreira, C., et al., *Biofouling control using microparticles carrying a biocide.* Biofouling, 2009. **26**(2): p. 205-212.
185. AFSSA. *Opinion on alternative methods for the chemical decontamination of carcasses. In French: Avis du 19 juin 2007 de l'Agence française de sécurité sanitaire des aliments relatif aux méthodes alternatives à la décontamination chimique des carcasses.* 2007. Maisons-Alfort (France).
186. Maillard, J.-Y. and S.P. Denyer, *Emerging bacterial resistance following biocide exposure: should we be concerned?* Chimica oggi, 2009. **27**(3): p. 26-28.
187. Mazzola, P.G., et al., *Minimal inhibitory concentration (MIC) determination of disinfectant and/or sterilizing agents.* Brazilian Journal of Pharmaceutical Sciences, 2009. **45**(2): p. 241-248.
188. Hegstad, K., et al., *Does the wide use of quaternary ammonium compounds enhance the selection and spread of antimicrobial resistance and thus threaten our health?* Microbial drug resistance, 2010. **16**(2): p. 91-104.
189. Gilbert, P. and L. Moore, *Cationic antiseptics: diversity of action under a common epithet.* Journal of Applied Microbiology, 2005. **99**(4): p. 703-715.
190. Monte, J., et al., *Antimicrobial Activity of Selected Phytochemicals against Escherichia coli and Staphylococcus aureus and Their Biofilms.* Pathogens, 2014. **3**(2): p. 473-498.
191. Jacoby, G.A., *Mechanisms of resistance to quinolones.* Clinical Infectious Diseases, 2005. **41**(Supplement 2): p. S120-S126.
192. Padrón, J.M., *Antimicrobial Drug Resistance (2 volumes). Edited by Douglas L. Mayers.* ChemMedChem, 2010. **5**(6): p. 960-961.
193. EUCAST, *The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MIC and zone diameters, version 4.0, 2014*, 2014.
194. CLSI, *Performance Standards for Antimicrobial Susceptibility Testing: Seventeenth Informational Supplement.*, in *CLSI document M100-S17*, C.a.L.S. Institute, Editor 2007: Wayne, Pennsylvania.
195. Tabata, A., et al., *Correlation between Resistance of Pseudomonas aeruginosa to Quaternary Ammonium Compounds and Expression of Outer Membrane Protein OprR.* Antimicrobial Agents and Chemotherapy, 2003. **47**(7): p. 2093-2099.
196. Bore, E., et al., *Adapted tolerance to benzalkonium chloride in Escherichia coli K-12 studied by transcriptome and proteome analyses.* Microbiology, 2007. **153**(4): p. 935-946.
197. Brazas, M.D. and R.E.W. Hancock, *Ciprofloxacin Induction of a Susceptibility Determinant in Pseudomonas aeruginosa.* Antimicrobial Agents and Chemotherapy, 2005. **49**(8): p. 3222-3227.
198. Becnel Boyd, L., et al., *Relationships among Ciprofloxacin, Gatifloxacin, Levofloxacin, and Norfloxacin MICs for Fluoroquinolone-Resistant Escherichia coli Clinical Isolates.* Antimicrobial Agents and Chemotherapy, 2009. **53**(1): p. 229-234.
199. French, G.L., *Bactericidal agents in the treatment of MRSA infections—the potential role of daptomycin.* Journal of Antimicrobial Chemotherapy, 2006. **58**(6): p. 1107-1117.
200. Woods, G.L. and J.A. Washington, *The clinician and the microbiology laboratory.* Mandell G, Bennett J, Dolin R, eds. Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases, 1995: p. 169-199.

201. Hancock, R.E., *Resistance mechanisms in Pseudomonas aeruginosa and other nonfermentative gram-negative bacteria.* Clinical Infectious Diseases, 1998. **27**(Supplement 1): p. S93-S99.
202. EU, *Regulation (EU) No. 528/2012 of the European Parliament and of the Council of 22 May 2012 concerning the making available on the market and use of biocidal products.* Official Journal of the European Union, Legislation 167 27.6. 2012, 2012: p. 1-123.
203. McDonnell, G. and A.D. Russell, *Antiseptics and Disinfectants: Activity, Action, and Resistance.* Clinical Microbiology Reviews, 1999. **12**(1): p. 147-179.
204. Myers, F., *Biocidal agents: modes of action and correlation with antibiotic resistance.* BIOMEDICAL SCIENTIST, 2008. **52**(3): p. 227.
205. Walton, J., et al., *Investigation into the effect of detergents on disinfectant susceptibility of attached Escherichia coli and Listeria monocytogenes.* Journal of Applied Microbiology, 2008. **105**(1): p. 309-315.
206. Jansen, A.C., *An investigation of resistance to quaternary ammonium compound disinfectants in bacteria,* 2012, University of the Free State Bloemfontein 9300 South Africa.
207. Hollyoak, V., P. Boyd, and R. Freeman, *Whirlpool baths in nursing homes: use, maintenance, and contamination with Pseudomonas aeruginosa.* Communicable disease report. CDR review, 1995. **5**(7): p. R102-4.
208. Baumgartner, A. and M. Grand, *Bacteriological quality of drinking water from dispensers (coolers) and possible control measures.* Journal of Food Protection®, 2006. **69**(12): p. 3043-3046.
209. Mena, K.D. and C.P. Gerba, *Risk assessment of Pseudomonas aeruginosa in water*, in *Reviews of Environmental Contamination and Toxicology Vol 201*2009, Springer. p. 71-115.
210. Adair, F.W., S.G. Geftic, and J. Gelzer, *Resistance of Pseudomonas to Quaternary Ammonium Compounds II. Cross-Resistance Characteristics of a Mutant of Pseudomonas aeruginosa.* Applied Microbiology, 1971. **21**(6): p. 1058-1063.
211. Carson, R.T., et al., *Use of antibacterial consumer products containing quaternary ammonium compounds and drug resistance in the community.* Journal of Antimicrobial Chemotherapy, 2008. **62**(5): p. 1160-1162.
212. Buffet-Bataillon, S., et al., *Effect of higher minimum inhibitory concentrations of quaternary ammonium compounds in clinical E. coli isolates on antibiotic susceptibilities and clinical outcomes.* Journal of Hospital Infection, 2011. **79**(2): p. 141-146.
213. Lamont, I.L., et al., *Siderophore-mediated signaling regulates virulence factor production in Pseudomonas aeruginosa.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(10): p. 7072-7077.
214. Raymond, K.N., E.A. Dertz, and S.S. Kim, *Enterobactin: An archetype for microbial iron transport.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **100**(7): p. 3584-3588.
215. Thomas, L., et al., *Development of resistance to chlorhexidine diacetate in Pseudomonas aeruginosa and the effect of a'residual'concentration.* Journal of Hospital Infection, 2000. **46**(4): p. 297-303.
216. Levy, S.B., *Antibacterial household products: cause for concern.* Emerging infectious diseases, 2001. **7**(3 Suppl): p. 512.
217. Chung, H.J., W. Bang, and M.A. Drake, *Stress Response of Escherichia coli.* Comprehensive Reviews in Food Science and Food Safety, 2006. **5**(3): p. 52-64.

218. Capita, R., et al., *Exposure to sub-lethal concentrations of food-grade biocides influences the ability to form biofilm, the resistance to antimicrobials and the ultrastructure of Escherichia coli ATCC 12806.* Applied and Environmental Microbiology, 2013: p. AEM. 02283-13.
219. Maillard, J.-Y., *Antimicrobial biocides in the healthcare environment: efficacy, usage, policies, and perceived problems.* Therapeutics and clinical risk management, 2005. **1**(4): p. 307.
220. Pankey, G. and L. Sabath, *Clinical relevance of bacteriostatic versus bactericidal mechanisms of action in the treatment of Gram-positive bacterial infections.* Clinical Infectious Diseases, 2004. **38**(6): p. 864-870.
221. Gomez Escalada, M., et al., *Triclosan–bacteria interactions: single or multiple target sites?* Letters in applied microbiology, 2005. **41**(6): p. 476-481.
222. Lambert, R. and J. Pearson, *Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non‐inhibitory concentration (NIC) values.* Journal of Applied Microbiology, 2000. **88**(5): p. 784-790.
223. Ioannou, C.J., G.W. Hanlon, and S.P. Denyer, *Action of Disinfectant Quaternary Ammonium Compounds against Staphylococcus aureus.* Antimicrobial Agents and Chemotherapy, 2007. **51**(1): p. 296-306.
224. Thomas, L., A. Russell, and J.Y. Maillard, *Antimicrobial activity of chlorhexidine diacetate and benzalkonium chloride against Pseudomonas aeruginosa and its response to biocide residues.* Journal of Applied Microbiology, 2005. **98**(3): p. 533-543.
225. Malek, S.A. and Y. Badran, *Pseudomonas aeruginosa PAO1 adapted to 2-phenoxyethanol shows cross-resistance to dissimilar biocides and increased susceptibility to antibiotics.* Folia microbiologica, 2010. **55**(6): p. 588-592.
226. Loughlin, M.F., M.V. Jones, and P.A. Lambert, *Pseudomonas aeruginosa cells adapted to benzalkonium chloride show resistance to other membrane-active agents but not to clinically relevant antibiotics.* Journal of Antimicrobial Chemotherapy, 2002. **49**(4): p. 631-639.
227. Pagedar, A., J. Singh, and V.K. Batish, *Efflux mediated adaptive and cross resistance to ciprofloxacin and benzalkonium chloride in Pseudomonas aeruginosa of dairy origin.* Journal of Basic Microbiology, 2011. **51**(3): p. 289-295.
228. Gilbert, P. and A.J. McBain, *Potential impact of increased use of biocides in consumer products on prevalence of antibiotic resistance.* Clinical Microbiology Reviews, 2003. **16**(2): p. 189-208.
229. Mc Cay, P.H., A.A. Ocampo-Sosa, and G.T. Fleming, *Effect of subinhibitory concentrations of benzalkonium chloride on the competitiveness of Pseudomonas aeruginosa grown in continuous culture.* Microbiology, 2010. **156**(1): p. 30-38.
230. Tezel, U. and S.G. Pavlostathis, *Quaternary ammonium disinfectants: microbial adaptation, degradation and ecology.* Current opinion in biotechnology, 2015. **33**: p. 296-304.
231. Bolla, J.-M., et al., *Strategies for bypassing the membrane barrier in multidrug resistant Gram-negative bacteria.* FEBS Letters, 2010. **585**(11): p. 1682-1690.
232. Johnston, H.J., et al., *A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity.* Critical reviews in toxicology, 2010. **40**(4): p. 328-346.
233. Wei, L., et al., *Silver nanoparticles: synthesis, properties, and therapeutic applications.* Drug discovery today, 2015. **20**(5): p. 595-601.

234. Liu, Z., H. Bai, and D.D. Sun, *Hierarchical CuO/ZnO membranes for environmental applications under the irradiation of visible light.* International journal of photoenergy, 2011. **2012**.
235. Rahimpour, A., et al., *Structural and performance properties of UV-assisted TiO 2 deposited nano-composite PVDF/SPES membranes.* Desalination, 2012. **285**: p. 31-38.
236. Wirtanen, G., M. Saarela, and T. Mattila-Sandholm, *Biofilms–Impact on hygiene in food industries*, 2000, Wiley. p. 327-372.
237. Maree, J.P., et al., *Recovery of Calcium Carbonate from Wastewater Treatment Sludge Using a Flotation Technique.* Journal of Chemical Engineering & Process Technology, 2012. **3**(130).
238. Qu, X., P.J.J. Alvarez, and Q. Li, *Applications of nanotechnology in water and wastewater treatment.* Water Research, 2013. **47**(12): p. 3931-3946.
239. Dean-Raymond, D. and M. Alexander, *Bacterial metabolism of quaternary ammonium compounds.* Applied and Environmental Microbiology, 1977. **33**(5): p. 1037-1041.
240. Boethling, R.S., *Environmental fate and toxicity in wastewater treatment of quaternary ammonium surfactants.* Water Research, 1984. **18**(9): p. 1061-1076.
241. Tapias, G., et al., *Interactions between cationic vesicles and Escherichia coli.* Langmuir, 1994. **10**(10): p. 3461-3465.
242. Campanhã, M., E. Mamizuka, and A. Carmona-Ribeiro, *Interactions between cationic liposomes and bacteria: the physical-chemistry of the bactericidal action.* Journal of lipid research, 1999. **40**(8): p. 1495-1500.
243. Vieira, D.B., L.F. Pacheco, and A.M. Carmona-Ribeiro, *Assembly of a model hydrophobic drug into cationic bilayer fragments.* Journal of colloid and interface science, 2006. **293**(1): p. 240-247.
244. Pereira, E.M., et al., *Hybrid materials from intermolecular associations between cationic lipid and polymers.* The Journal of Physical Chemistry B, 2008. **112**(31): p. 9301-9310.
245. Melo, L.c.D., E.M. Mamizuka, and A.M. Carmona-Ribeiro, *Antimicrobial particles from cationic lipid and polyelectrolytes.* Langmuir, 2010. **26**(14): p. 12300-12306.
246. Naves, A.F., et al., *Antimicrobial particles from emulsion polymerization of methyl methacrylate in the presence of quaternary ammonium surfactants.* Langmuir, 2013. **29**(31): p. 9677-9684.

# Appendix

## A. SD calculation

The results were obtained from crossing results from two consecutive days. The results treatment was done by calculating average of $OD_{610nm}$ registered for each concentration along with respective standard error (s). The final values were subjected to a *s* with the following formula:

$$s = \sqrt{\sigma_1^2 - \sigma_2^2} \qquad \text{[Eq. A.1-1]}$$

Where, $\sigma_1$ that defines the standard deviation of the O.D. replicates done for each concentration tested and, $\sigma_2$ corresponds to standard deviation of O.D. replicates registered for TSB.

## B. Supplementary data

Quantification of BDMDAC concentration adhered to the surface of $CaCO_3$-PEI|PSS|BDMDAC MPs

The method applied for the determination of antimicrobial amount adhered to functionalized MPs was already validated by Ferreira, Pereira [171]. The assays performed with HPLC system (described at section 3.5.4) detected a concentration of 0.7 mg of BDMDAC per mL of solution in resulted supernatant after process of incorporation of antimicrobial in MPs. The concentration of biocide adhered to the particles was assessed as the difference between the biocide concentration in solution before (1000 mg/L) and after incorporation in the particles.

As a result, the final concentration of 300 mg of BDMDAC per liter resuspended in 10 mL of 0.1M $H_3BO_3$ solution, at pH 9, represents 'coat-antimicrobial' concentration obtained and used as the theoretical value used in the further assays.

- *Estimative of BDMDAC amount adhered to each microparticle*

As total mass defined by protocol, was weighed 0.09 g of $CaCO_3$ MPs at powder form. With spherical shape confirmed by SEM images, volume of each MP can be defined through following formula:

$$V_{sphere} = \frac{4}{3}\pi r^3 \qquad \text{[Eq. B.1-1]}$$

For determination of mass of a single MP is necessary to apply the density formula:

$$\rho = \frac{m}{V} \qquad \text{[Eq. B.1-2]}$$

With a MP diameter around 3 µm (=$3\times10^{-4}$ cm) and a MP density of 2.83 g/cm$^3$, the mass of a single MP is $4.001\times10^{-11}$ g. In terms of number of particles present in each stock

suspension, with a total of 0.09 g of mass of the particles weighed for stock suspension, is obtained about $2.25\times10^9$ particles.

The amount of BDMDAC coated at MPs surface is obtained through the ratio established between total amount of BDMDAC present in stock suspension and number of particles present in each 10 mL of total stock suspension:

$$m_{BDMDAC\,/MP} = \frac{total\ of\ BDMDAC\ mass\ present\ \times\ total\ volume\ of\ stock\ solution}{number\ of\ particles\ in\ stock\ solution} \quad \text{[Eq. B.1-3]}$$

As result was obtained a total of BDMDAC of:

$$m_{BDMDAC\,/MP} = \frac{0.3 \times 10}{2.25 \times 10^9} = 1.33 \times 10^{-9}\ mg\ of\ BDMDAC/particule$$