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Inactivação de biofilmes de *Pseudomonas spp.* com fotossessibilizadores naturais e sintéticos

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Maria Ângela Sousa Dias Alves da Cunha, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

Aos meus pais, avós e madrinha

o júri

presidente	**Prof. Doutor António José Arsénia Nogueira** Professor Associado c /agregação do Departamento de Biologia da Universidade de Aveiro
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palavras-chave

Inativação fotodinâmica, biofilmes, *Pseudomonas spp.*, curcumina, porfirina tetra catiónica.

resumo

As porfirinas catiónicas têm sido muito utilizadas como fotossensibilizadores (PSs) na inativação de microrganismos tanto na forma planctónica como em biofilmes. No entanto, a eficiência da curcumina, um PS natural, na inativação de biofilmes está ainda muito pouco estudada.

Os objetivos deste trabalho foram: (1) avaliar e comparar a eficiência de uma porfirina tetra catiónica (Tetra-Py^{+}-Me) e da curcumina na inativação fotodinâmica de biofilmes de *Pseudomonas spp.*, bem como na inativação de células na forma planctónica; (2) avaliar o efeito destes PSs nas fases de adesão e maturação do biofilme. Em experiências de erradicação, biofilmes de *Pseudomonas spp* aderentes a tubos de silicone foram sujeitos a irradiação com luz branca (180 J cm^{-2}) na presença de diferentes concentrações (5 e 10 μM) de PS. Em experiências de colonização, os suportes sólidos foram imersos em suspensões de células, adicionados de PS e irradiados durante a fase de adesão (864 J cm^{-2}). Após transferência dos suportes sólidos para novo meio contendo idêntica concentração de PS, prosseguiu-se com a irradiação (2592 J cm^{-2}) durante a fase de maturação dos biofilmes. As experiências de inativação da forma planctónica foram conduzidas em suspensões de células, adicionadas de concentrações de PS equivalentes às usadas nas experiências com biofilmes. A inativação de células livres e de biofilmes (experiências de erradicação e de colonização) foi avaliada por quantificação de células viáveis através de sementeira em meio sólido, antes e depois da irradiação. Os resultados demonstraram que a porfirina Tetra-Py^{+}-Me inativou eficazmente quer as células planctónicas (3.7 e 3.0 log), quer os biofilmes de *Pseudomonas spp* (3.2 e 3.6 log). Nos ensaios de colonização, reduziu em cerca de 2.2 log a concentração de células aderentes e, durante a fase de maturação, causou uma inativação de 5.2 log na concentração de células viáveis. A curcumina revelou-se um fotossensibilizador muito pouco eficaz na inactivação de células planctónicas (0.7 e 0.9 log) e por essa razão não foi testada nos ensaios de erradicação. Nos ensaios de colonização, não afetou a adesão e causou uma redução muito modesta (1.0 log) na concentração de células durante a fase de maturação.

Os resultados confirmam que a inativação fotodinâmica é uma estratégia promissora no controle de biofilmes instalados e na prevenção da colonização. A curcumina, no entanto, não representa uma alternativa vantajosa às porfirinas, no caso dos biofilmes de *Pseudomonas spp.*

keywords

Photodynamic inactivation, biofilms, Pseudomonas spp., curcumin, tera-cationic porphyrin.

abstract

Cationic porphyrins have been widely used as photosensitizers (PSs) in the inactivation of microorganisms, both in biofilms and in planktonic forms. However, the application of curcumin, a natural PS, in the inactivation of biofilms, is poorly studied. The objectives of this study were (1) to evaluate and compare the efficiency of a cationic porphyrin tetra (Tetra-Py^{+}-Me) and curcumin in the photodynamic inactivation of biofilms of *Pseudomonas* spp and the corresponding planktonic form; (2) to evaluate the effect of these PSs in cell adhesion and biofilm maturation. In eradication assays, biofilms of *Pseudomonas spp* adherent to silicone tubes were subjected to irradiation with white light (180 J cm^{-2}) in presence of different concentrations (5 and 10 μM) of PS. In colonization experiments, solid supports were immersed in cell suspensions, PS was added and the mixture experimental setup was irradiated (864 J cm^{-2}) during the adhesion phase. After transference solid supports to new PS-containing medium, irradiation (2592 J cm^{-2}) was resumed during biofilm maturation. The assays of inactivation of planktonic cells were conducted in cell suspensions added of PS concentrations equivalent to those used in experiments with biofilms. The inactivation of planktonic cells and biofilms (eradication and colonization assays) was assessed by quantification of viable cells after plating in solid medium, at the beginning and at the end of the experiments. The results show that porphyrin Tetra-Py^{+}-Me effectively inactivated planktonic cells (3.7 and 3.0 log) and biofilms of *Pseudomonas spp* (3.2 and 3.6 log). In colonization assays, the adhesion of cells was attenuated in 2.2 log, and during the maturation phase, a 5.2 log reduction in the concentration of viable cells was observed. Curcumin failed to cause significant inactivation in planktonic cells (0.7 and 0.9 log) and for that reason it was not tested in biofilm eradication assays. In colonization assays, curcumin did not affect the adhesion of cells to the solid support and caused a very modest reduction (1.0 log) in the concentration of viable cells during the maturation phase.

The results confirm that the photodynamic inactivation is a promising strategy to control installed biofilms and in preventing colonization. Curcumin, however, does not represent an advantageous alternative to porphyrins in the case of biofilms of *Pseudomonas spp*.

Table of Contents

List of acronyms and abbreviations

CFU	Colony forming units
DC	Dark control
DMSO	Dimethyl sulfoxide
EPS	Extracellular polymeric substances
Gram (+)	Gram-positive
Gram (-)	Gram-negative
LC	Light control
OD	Optical density
PBS	Phosphate buffered saline
PDI	Photodynamic Inactivation
PS	Photosensitizer
ROS	Reactive oxygen species
TSA	Tryptic soy agar
TSB	Trypic soy broth

1. Introduction

1.1. Microbial biofilms

Biofilms form predominantly in aqueous media, attached to a solid surface (substratum), but they can also be found at interfaces (air-water, solid-liquid, solid-air). In biofilms, cell density is high (10^{10} cells mL^{-1} of hydrated biofilm) (Traba *et al.*, 2013), multiple species co-exist (Traba *et al.*, 2013) and a wide range of physical, metabolic and chemical heterogeneities occur (Costerton *et al.*, 1987; Beer and Stoodley, 2006; Denkhaus *et al.*, 2007). During biofilm development there is a coordinated phenotypic shift and therefore, cells in biofilm are different from free living cells (Denkhaus *et al.*, 2007).

Despite the fact that traditional microbiology is mostly based on the study of planktonic cells, biofilms have increasingly gained importance as the primary habitat for many microorganisms since it is currently recognized that only a small fraction of bacteria in natural ecosystems exists in planktonic state, and that biofilms are, in fact, the predominant state of bacteria in nature (Davey and O'toole, 2000).

Biofilms, flocks, and other microbial aggregates are abundant in natural environments. Biofilms are ubiquitous in nature, covering the surface of rocks and plants, sediment surfaces in seawater and freshwater systems, especially under extreme conditions of temperature and salinity (Beer and Stoodley, 2006). Flocks are fragile structures suspended in fresh and seawater (called river- or marine snow) and typically develop up during bloom periods after an increased input of nutrients (Beer and Stoodley, 2006; Denkhaus *et al.*, 2007). Microbial cells associated with sediment and suspended in flocks or aggregates, even though different in appearance from conventional biofilms, have many important features in common and thus are included in the definition of biofilm (Costerton *et al.*, 1987, 1995).

Using the simplistic definition proposed by (Carpentier and Cerf, 1993) a biofilm is "a community of microbes embedded in an organic polymer matrix, adhering to a surface". A more comprehensive definition proposed by (Elder *et al.*, 1995) describes a biofilms as "a functional consortium of microorganisms organized within an extensive

exopolymer matrix". More recently, (Lear and Lewis, 2012) defined biofilm as microbial aggregates in which cells adhere to each other on a surface surrounded by a self-produced matrix of extracellular polymeric substances.

Biofilms are mainly composed of water, microbial cells, that account for less than 10% of the dry mass, and extracellular polymeric substances (EPS), that represents over 90% of the biofilm dry mass and are often referred as its main constituent **(Table 1).** Depending on the environment and on the structure of the microbial community, biofilms can also contain variable amounts of trapped particles and dissolved substances of organic or inorganic nature (Denkhaus *et al.*, 2007; Nadell *et al.*, 2009; Flemming and Wingender, 2010).

Table 1| Overall composition of microbial biofilms (adapted from Denkhaus *et al.*, 2007)

Extracellular polymeric substances (EPS)
Cationic groups in amino sugars and proteins (e.g. NH_3^+); Anionic groups in uronic acids, proteins, and nucleic acids (e.g.COO^-; $HPO_4^{-)}$; Apolar groups from proteins (such as in aromatic amino acids), (phospho) lipids and humic substances.
Microbial cell
Outer membrane: lipopolysaccharides of Gram-negative cells (cell wall consisting of *N*-acetylglucosamine and *N*-acetylmuramic acid, offering cationic and anionic sites, and the lipoteichoic acids in gram-positive cells); Cytoplasmic membrane, offering a lipophilic region (cytoplasm, as a water phase separated from the surrounding water).
Minerals
Precipitates (sulfides, carbonates, phosphates, hydroxides); Free and bound metals (Ca_2^+, Fe_3^+, Mg_2^+).
Environmentally relevant substances
Organic pollutants (e.g. biocides, detergents, xenobiotics); Inorganic pollutants (e.g. heavy metals).

1.2. The biofilm matrix

The extracellular matrix corresponds to the materials that are produced by microorganisms, in which cells are embedded, and can comprise approximately 50-90% of the total organic matter content (Flemming and Wingender, 2010). EPS consist of a conglomeration of different types of biopolymers that forms the scaffold for the three-dimensional biofilm architecture (the dense areas, pores and channels) and it is responsible for adhesion to surfaces and for cohesion in the biofilm (Branda *et al.*, 2005; Vu *et al.*, 2009). EPS determine the immediate conditions of life of biofilm cells by affecting porosity, density, water content, charge, sorption properties, hydrophobicity, and mechanical resistance of the biofilm (Nadell *et al.*, 2009; Flemming and Wingender, 2010). EPS are also a source of nutrients, although some components of EPS are only slowly biodegradable (Flemming and Wingender, 2010). The matrix works as a defensive barrier that protects cells against desiccation, oxidizing or charged biocides, some antibiotics and metallic cations, ultraviolet radiation and host immune defenses **(Table 2).** The matrix also acts as a recycling centre by keeping all of the components of lysed cells available. This includes DNA, which may represent a reservoir of genes for horizontal gene transfer (Prakash *et al.*, 2003).

EPS are usually classified as capsular polysaccharides or exopolysaccharides. The difference between them are that when bacteria are grown in shaken liquid cultures and then collected by centrifugation, extracellular polysaccharides that remain cell-associated are referred to as the capsule, while those remaining in the supernatant are denominated exopolysaccharides (Branda *et al.*, 2005). EPS are primarily composed of polysaccharides, but the overall chemical composition of the EPS pool may vary significantly under different physical and chemical conditions **(Table 2).** Some of them are neutral or polyanionic, as is the case for the EPS of gram-negative bacteria. The presence of uronic acids (D-glucuronic, D-galacturonic, and D-mannuronic) or ketal-linked pryruvates confers the anionic character. Another fraction of EPS is composed by exopolysaccharides, including alginate, xanthan and colanic acid that are polyanionic (Sutherland, 2001; Nadell *et al.*, 2009; Flemming and Wingender, 2010). These properties are important because they allow the association of divalent cations such as calcium and magnesium, which have

been shown to cross-link with the polymer strands and provide greater binding force in a developed biofilm (Sutherland, 2001).

In *Pseudomonas aeruginosa* biofilms, the matrix is a defined mixture of polysaccharides, membrane vesicles, fimbriae, nucleic acids and proteins (Karatan and Watnick, 2009). The biofilm structure in *P. aeruginosa* is mainly maintained by three exopolysaccharides, Psl, Pel, and alginate (Branda *et al.*, 2005; Franklin *et al.*, 2011). The Psl exopolysaccharide is composed of mannose, galactose, rhamnose, glucose, and trace amounts of xylose and it is vital for bacterial cells adherence to a substratum and biofilm structure (Ma *et al.*, 2006; Ryder *et al.*, 2007; Franklin *et al.*, 2011; Wei and Ma, 2013). The Pel structure is still unknown and further biochemical analyses are necessary. However, it is thought that Pel is also involved in the cohesion of the extracellular matrix (Wei and Ma, 2013). Alginate, an acetylated polymer composed of nonrepetitive monomers of β-1,4 linked L-guluronic and D-mannuronic acids, is a capsular polysaccharide (virulence factor) that confers a selective advantage for *Pseudomonas aeruginosa* in the cystic fibrosis. (Franklin *et al.*, 2011). Alginates protects *P. aeruginosa* from the inflammation process, once it captures free radicals, released by activated macrophages, and provides protection from phagocytes (Ryder *et al.*, 2007; Franklin *et al.*, 2011; Wei and Ma, 2013).

In addition to exopolysaccharides, extracellular DNA (eDNA) is also an important component of the *P. aeruginosa* biofilm matrix, originating from random chromosomal DNA, which functions as a cell-to-cell inter-connecting component in the biofilm (Allesen-holm *et al.*, 2006; Flemming and Wingender, 2010). In the biofilm matrix, eDNA contributes to cation gradients, genomic DNA release and inducible antibiotic resistance (Mulcahy *et al.*, 2008). The existence of non-enzymatic cell surface-associated proteins (lectins LecA and fucose-specific lectin LecB) in the matrix of *P. aeruginosa*, proved to be involved in the formation and stabilization of the polysaccharide matrix network, constituting a link between the bacterial surface and the EPS (Flemming and Wingender, 2010; Wei and Ma, 2013).

Table 2| Principal functions of extracellular polymeric substances in bacterial biofilms, (adapted from Flemming and Wingender, 2010).

Function	Relevance for biofilms	EPS components involved
Adhesion	Allows the initial steps in the colonization of abiotic and biotic surfaces by planktonic cells, and the long-term attachment of whole biofilms to surfaces.	Polysaccharides, proteins, eDNA and amphiphilic molecules
Aggregation of bacterial cells	Enables bridging between cells, the temporary immobilization of bacterial populations, the development of high cell densities and cell–cell recognition.	Polysaccharides, proteins and eDNA
Cohesion of biofilms	Forms a hydrated polymer network (the biofilm matrix), mediating the mechanical stability of biofilms (often in conjunction with multivalent cations) and, through the EPS structure (capsule, slime or sheath), determining biofilm architecture, as well as allowing cell–cell communication.	Neutral and charged polysaccharides, proteins (such as amyloids and lectins), and eDNA
Retention of water	Maintains a highly hydrated microenvironment around biofilm organisms, leading to their tolerance of desiccation in water-deficient environments.	Hydrophilic polysaccharides and, possibly, proteins
Protective barrier	Confers resistance to nonspecific and specific host defenses during infection, and confers tolerance to various antimicrobial agents (for example, disinfectants and antibiotics), as well as protecting cyanobacterial nitrogenase from the harmful effects of oxygen and protecting against some grazing protozoa.	Polysaccharides and proteins

1.3. Life cycle of biofilm communities

In general, biofilms can be formed practically in all surfaces being the most "easy to stick" surfaces, the ones that are rougher, coated and hydrophobic (Salta *et al.*, 2013).

The “standard model” of biofilms life cycle **(Fig.1.1)** is described in five steps: (1) reversible attachment of bacteria to a surface (by migration or division of sessile cells to cover an empty region of the surface); (2) production of EPS that allows cells to adhere permanently to the substrate; (3) formation of micro-colonies or colonization; (4) maturation corresponding to development of a mature, spatially structured biofilm via a complex process involving additional EPS production, signaling, cellular motility, reproduction, and the expression of biofilm-specific properties (such as antibiotic resistance); (5) dispersal or detachment, during which the "free swimming cells" are released and the colonization process will be initiated elsewhere (Monds and Toole, 2009).

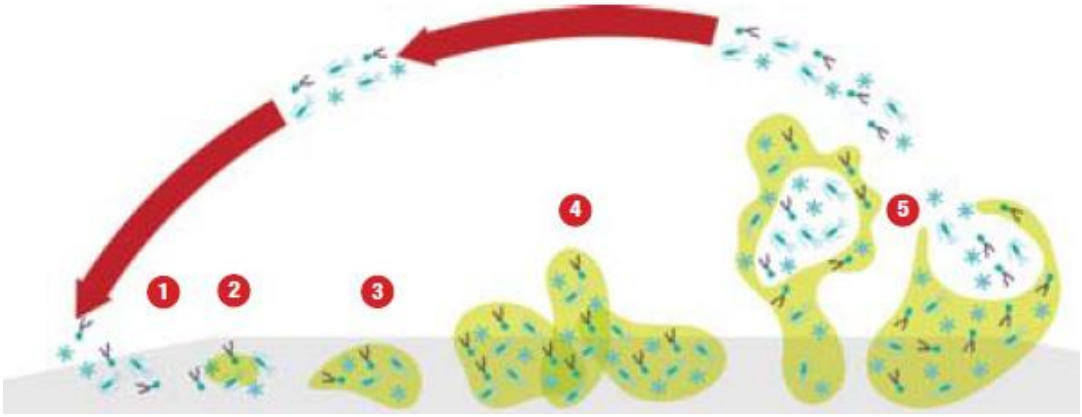


Fig. 1.1 - The biofilm life cycle: 1: individual cells populate the surface; 2: EPS is produced and attachment becomes irreversible; 3 and 4: biofilm develops and matures; 5: single cells are detached and released from the biofilm (adapted from Abelson and McLaughlin, 2012)**.**

1.4. Quorum sensing: cell-to-cell communication

It is currently accepted that bacteria are highly interactive and exhibit several social behaviours, such as pullulate motility, conjugal plasmid transfer, antibiotic resistance, symbiosis, sporulation, biofilm formation and even virulence (Turovskiy *et al.*, 2007). These processes are mainly regulated by quorum sensing systems described as the phenomenon whereby the accumulation of ‘signalling’ molecules (autoinducers) in the surrounding environment enables a single cell to sense the number of bacteria (cell

density), so that the population as a whole can make a coordinated response (Turovskiy *et al.*, 2007). Quorum sensing is based in three principles: first, the production of autoinducers (AIs), which are the signaling molecules, by the bacteria. Secondly, AIs are detected by receptors that exist in the cytoplasm or in the membrane. Finally, in order to activate the necessary gene expression for cooperative behaviors, the detection of AIs results in activation of AIs production (Rutherford and Bassler, 2012). At low cell density, AIs diffuse away, and, therefore, are present at concentrations below the threshold required for detection. At critical cell densities, the binding of a regulator protein to the signal leads to the switch on of genes controlled by quorum sensing and, therefore, a synchronized population response (Miller and Bassler, 2001).

Bacteria use several AIs to communicate within and between bacterial species. Acylated homoserine lactones (AHL) are normally used by Gram-negative bacteria and processed oligopeptides are generally used by Gram-positive bacteria to communicate (Miller and Bassler, 2001). In several species of bacteria, disruption of the quorum sensing system has been shown to affect biofilm formation and differentiation. For example, in *Pseudomonas aeruginosa,* the inactivation of quorum sensing, causes the loss of biofilm structure (Turovskiy *et al.*, 2007).

1.5. Advantages of the sessile lifestyle

Biofilms communities obtain a great number of benefits by the way how bacteria interact with each other, once they are better adapted to withstand nutrient deprivation, pH changes and oxygen radicals. They also develop defense mechanisms (physical forces such as the shear forces and phagocytosis) or antibiotic resistance, that help them to endure and thrive as long as they stick together (Mah and O'Toole, 2001; Kreft, 2004).

1.5.1. Physiological cooperation

A biofilm community tends to represent a benefit for all its members.

Cooperation is a phenotypic behaviour that may benefit an individual or a population (Gestel *et al.*, 2015). In a population, not all individuals are cooperative but all can get access to the benefit that some may produce, without paying the costs. For example, in a rich environment, *Pseudomonas aeruginosa* produce siderophores (iron-

scavenging molecules) that are beneficial when iron is limiting, showing a faster growth than the mutant strains unable to express siderophores. Notwithstanding, siderophore production has its costs so, in a mixed population of wild type and a mutant strain, the mutants can get the benefit of siderophore production without paying the cost, and therefore increase in frequency and gaining selective advantage (West *et al.*, 2006; Gestel *et al.*, 2015).

Metabolic cooperation is often referred to as metabolic commensalism and happens when one species metabolizes nutrients and releases reaction products that are used by another species (Elias and Banin, 2012). For example, in a mixed biofilm of *Pseudomonas putida* and *Acinetobacter*, the latest produces benzoate that is then metabolized by *P. putida*. It is also known that *Acinetobacter* occurs in the upper layers of the biofilm, close to the nutrient source, while *P. putida* concentrates in the lower layers, benefiting from benzoate secreted from the *Acinetobacter* (Elias and Banin, 2012). In multispecies biofilms, benefits received may compensate the costs of the cooperative behaviour. For example, colonization of human teeth and the oral mucosa by early colonizers *Streptococcus oralis* and *Actinomyces naeslundii* suggest that cooperation between these species allows them to grow where neither can survive alone (West *et al.*, 2006).

1.5.2. Dormancy

In the midst of inhospitable conditions such as nutrient starvation and fluctuations in environment, abiotic factors such as temperature, osmotic pressure, light and pH, bacterial biofilm communities can enter into a viable but nonculturable state, which is referred as dormancy **(Fig.1.2).** Cells may regain viability when the biofilm has recovered its active development form and are able to reproduce (Oliver, 2010).

After entering the dormant state, microorganisms may display a series of characteristics such as, the differentiation of endospores, conidia and cysts. Another evident change is the reduction in cell size. "Dwarf cells" are common in nutrient depleted marine environments. They also exhibit reduced concentrations of DNA, lipids, fatty acids and proteins and an increase of reserves that are necessary for meeting the energy requirements for survival during that state (Lennon and Jones, 2011).

Although dormancy does not require as much energy as normal cellular activity, minimum energy is still required for repairing DNA and protein damage. The energy cost is also dependent on dormancy time. Some bacterial populations have a fast respond to the environment fluctuations and therefore the costs are almost inexistent. In other cases, microorganisms can be dormant and preserved in materials including amber and aquatic sediments for several thousand to several hundred million years (Lennon and Jones, 2011).

Persister cells, well represented in biofilms, are an example of spontaneously initiated dormancy, once they represent a subpopulation of bacterial cells that remains alive after being exposed to antibiotics but still sensitive to that antibiotic upon being re-grown and giving rise to the same small fraction of persisters (Lewis, 2007). Persister cells can be produced spontaneously or in response to starvation or resource limitation and serve as an important reservoir, or seed bank for cells that guarantee long-term population viability, saving the bacterial population from extinction (Lewis, 2007, 2010).

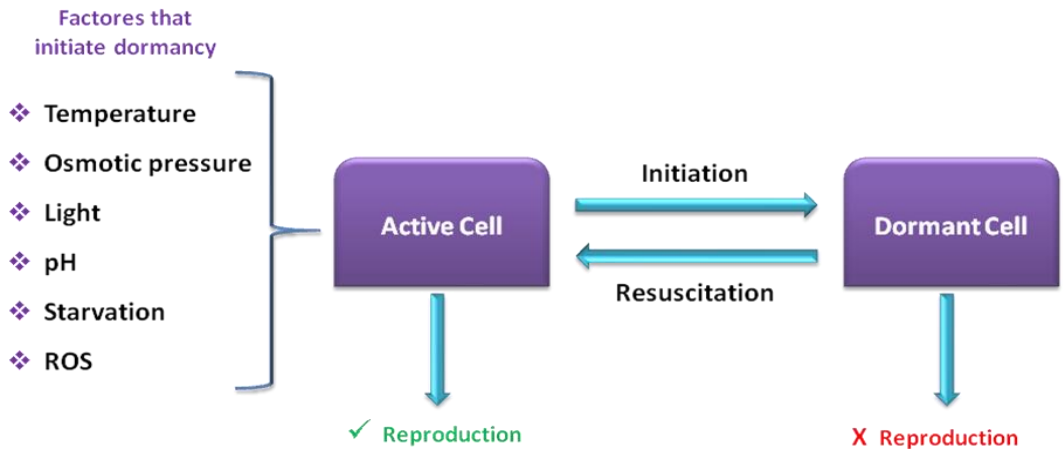


Fig. 1.2 - Dynamics of microbial dormancy (adapted from Lennon and Jones, 2011).

1.5.3. Antibiotic tolerance

Bacteria may form a biofilm in response to several factors, which may include cellular recognition of specific or non-specific attachment sites on a surface, nutritional signs, or in some cases, exposure to antibiotics (Hoffman *et al.*, 2005). The biofilm expansion allows the inner cells to become more resistance to antibiotics. In fact, depending on the organism and the type of antibiotic, bacteria can become a thousand times more resistance to antimicrobial stress than free swimming bacteria of the same species (Dunne, 2002). The interest in antimicrobial resistance and or enhanced tolerance to antimicrobials is increasing because the relation between biofilms and persistent infections is undoubtedly established (Bjarnsholt, 2013; Høiby *et al.*, 2015). The most frequent defense mechanisms against antibiotics, such as target mutations, low cell permeability, efflux pumps and modifying enzymes, do not appear to be the cause of the reduced antimicrobial susceptibility (Costerton *et al.*, 1999; Stewart, 2002). The main mechanisms of antibiotic resistance in biofilms do not seem to depend on plasmids, transposons, or mutations that confer innate resistance to individual bacterial cells. In biofilms, resistance appears to rely mostly on multicellular strategies (Stewart and Costerton, 2001; Parsek and Fuqua, 2004).

The main mechanisms involved in antibiotic tolerance of bacteria in biofilms are (1) reduced antibiotic penetration; (2) altered microenvironment and slow growth; (3) adaptive responses **(Fig.1.3)**. The first mechanism hypothesizes the possibility of slow or incomplete penetration of the antibiotic into the biofilm. In some cases the antibiotic penetration into biofilms have shown that some antibiotics readily infuse in bacterial biofilms, in other cases the antibiotic may be deactivated in the biofilm, which leads to a profoundly retarded penetration (Davies, 2003). For example, *Klebsiella pneumoniae* wild type biofilm has two different strains, one that is β-lactamase-negative that allows the penetration of ampicillin and subsequently its death, and other that is β-lactamase-positive that is not sensitive to ampicillin (Stewart and Costerton, 2001). The ability of the antibiotic to reach the substratum might be a function of biofilm surface coverage in addition to biofilm penetration, once it can pass through gaps in the interstices between microcolonies.

The second mechanism hypothesizes that the biofilm antibiotic tolerance depends on an altered chemical microenvironment within the biofilm. It is known that differences in nutrient and oxygen availability within the biofilm result in differences in metabolic activity among bacteria, leading to population heterogeneity (Davies, 2003). The majority of antibiotics has metabolically active cells as first targets. The biofilms metabolic heterogeneity leads to variation in susceptibility to antimicrobial agents. It has also been proposed that slow-growing and non-growing bacteria (dormant cells) contribute significantly to the decrease of biofilm susceptibility to antimicrobial agents (Drenkard, 2003).

The last mechanism hypothesized that bacterial biofilms are prepared to deal with stress resultant from environmental fluctuations, such as abrupt temperature changes, oxidative stress, low water activity, DNA damage and starvation. The sigma factor *rpoS* is a general stress response regulator that activates expression of a number of genes necessary to maintain cell viability during stationary phase when cells experience nutrient starvation. Due to nutrient limitation, biofilms would induce the expression of the *rpoS*, resulting in physiological changes that would mediate protection against environmental stress and antimicrobial agents (Stewart, 2002). Consistent with this hypothesis, (Xu *et al.*, 2001) reported that RpoS, was expressed at higher levels in *P. aeruginosa* biofilm cells, which did not happen in stationary-phase planktonic cultures.

The effectiveness of antibiotics is dependent on growth conditions and cell activity rates. Within biofilms, steep micro-gradients of concentration of key metabolic substrates and products occur. Due to these chemical gradients, biofilms promote dormant, slow-growing or stationary phase cells rather than exponentially growing cells (Stewart, 2002). In a well succeeded biofilm, bacteria are not all in the same growth phase and these heterogeneous populations become less susceptible to an antimicrobial attack (Høiby *et al.*, 2010). In less successful and more vulnerable biofilms, bacteria grow at a more uniform intermediate rate (Høiby *et al.*, 2010). Therefore fore, low activity rates represent a key factor of tolerance against antibiotics. In addition, bacteria are prepared to express a multitude of stress responses, in response to environmental fluctuations,

such as abrupt temperature changes, oxidative stress, low water activity, DNA damage and starvation.

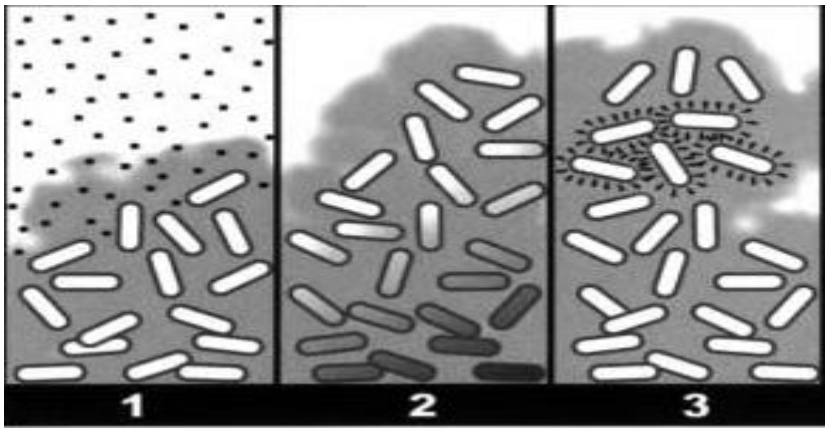


Fig.1.3 - Mechanisms of biofilm resistance: (1) the antibiotic (dots) penetrates slowly or incompletely; (2) a concentration gradient of a metabolic substrates or products leads to zones of slow or non-growing bacteria (shaded cells); (3) adaptive stress responses are expressed by some of the cells (marked cells) (adapted from, Stewart, 2002).

1.6. Biofilms and human affairs

Although biofilms may be interesting for biotechnological applications (Dvořák *et al.*, 2014) they are most often regarded as undesirable because of their relations with disease (Bjarnsholt, 2013), food safety (Brooks and Flint, 2008), biocorrosion (Deutzmann *et al.*, 2015), clogging of industrial filters and piping systems (Drescher *et al.*, 2013) with consequent human and material losses. It is estimated that 65% of all hospital infections are due to bacterial biofilms (Percival *et al.*, 2011). Cystic fibrosis patients have a high probability to develop *Pseudomonas aeruginosa* infections, which is very difficult to control, causing typically life-long chronic infections (Bjarnsholt, 2013). Tooth decay and periodontal disease, and chronic wound infections are some of other examples of infections caused by biofilms. Biofilms are an economical burden to biofilm infections with those involving medical devices estimated to cost $20 billion in the USA alone, being spend globally millions of dollars, in the control of industrial biofilms and in antifouling strategies (Römling *et al.*, 2014).

Biofilms are estimated to be responsible for 80% of all infections, in wide range of tissues and organs (Høiby *et al.*, 2011). Human diseases associated with biofilms are often related with the presence of some implantable medical device (e.g. catheters, joint

prostheses, heart valves) (McConoughey *et al.*, 2014) or occur as a consequence of some impairment of the host defense systems like lung infections in cystic fibrosis patients (Bjarnsholt, 2013; Römling *et al.*, 2014). In fact, the insertion of a foreign body that will inhabit in the patient for several years increases the risk of biofilm formation and proliferation. The most typical bacteria to cause periprosthetic infection include coagulase (-) staphylococci (30-43%), *Staphylococcus aureus* (12-23%), streptococci (9-10%), enterococci (3-7%) and Gram (-) bacteria (3-6%) (McConoughey *et al.*, 2014).

Chronic and surgical wounds are a significant and growing problem in healthcare today, only in USA, the treatment costs are estimated to be upwards of $20 billion-$25 billion annually (Seth *et al.*, 2012). The majority of chronic wound biofilms have been shown to consist of a mixed population of multiple bacterial species, being the most usual species such as *Serratia*, *Staphylococcus*, *Pseudomonas* and *Escherichia coli*, with one study demonstrating an average of 5.4 species of bacteria in each chronic human wound (Kennedy *et al.*, 2010). An important trait of the biofilms associated with infections is that, in the most cases, they consist of a single bacterial species. The exceptions to this overview are the biofilms associated with urinary catheters and prostheses which often consist of a variety of organisms (Römling *et al.*, 2014).

Biocorrosion or microbial corrosion is the damage caused or accelerated by the presence of microorganisms and their metabolic activities including enzymes, exopolymers, organic and inorganic acids, as well as volatile compounds such as ammonia or hydrogen sulfide (Beech and Gaylarde, 1999). Sulphate reducing and iron bacteria are well known examples of organisms whose biological activity or metabolic by-products cause biocorrosion. Such bacteria live in areas of low oxygen concentrations, for instance under a layer of aerobic fouling organisms, or in purged water such as that found in oil storage tanks and well flood water (Beech and Gaylarde, 1999; Beech *et al.*, 2005). It is a common problem in humid and aqueous environments, including ships hulls, propellers and sea water handling pipes (Salta *et al.*, 2013), important industrial activities such as petroleum exploration (Beech *et al.*, 2005) nuclear power plants, construction and shipping (Beech and Gaylarde, 1999). Biocorrosion is also a major issue for economic, health, safety, technological as well as environmental purposes, being spent every year,

globally thousands of dollars in attempt of resolving this problem (Beech and Gaylarde, 1999).

Food processing environments offer a set of amenable conditions for the settlement of biofilms, such as the availability of nutrients, moisture and the presence of microorganisms in raw products. Bacterial adhesion to food processing surfaces is a fast process, so thus it is necessary to clean and disinfect all the surfaces, and even that can be insufficient it avoid the adhesion of microorganisms (Kumar and Anand, 1998). Cleaning procedures only remove approximately 90% of attached bacteria from surfaces without actually killing them and disinfection is crucial to prevent biofilm development (Oliviera *et al.*, 2010).

Streptococcus thermophilus biofilms has been found on the pasteurized milk, once it is prone to attach to the heat exchangers in milk processing equipment (Srey *et al.*, 2013). *Bacillus cereus* spores have also been found in milk and meat, since they are hydrophobic they are drawn to the surfaces of the pipes in the process equipment (Srey *et al.*, 2013). *Listeria monocytogenes* and other human pathogens have been found in food processing industries working with meat, milk and other kinds of foods and has been detected in drains, condensed or stagnant water, floors and process equipment (Chmielewski and Frank, 2003; Srey *et al.*, 2013).

Pseudomonas are also found in food processing environments like drains and floors, fruits, vegetables, meat surfaces and in low acid daily products. *Pseudomonas spp*. produce copious amounts of EPS and has been shown to attach and form biofilms on stainless steel surfaces (Chmielewski and Frank, 2003). They also form biofilms within Listeria and Salmonella.

1.7. Strategies for biofilm control

In order to prevent the adhesion of microorganisms, delay biofilm development and eliminate, or at least reduce its viability, several strategies have been studied and implemented. The most common strategies for biofilm control involve physical, chemical or biological treatment, being the chemical approaches the most common and economically feasible (Ammons, 2010; Taraszkiewicz *et al.*, 2012; Tan *et al.*, 2014).

The application of some families of enzymes that depolymerize components of the biofilm, sodium salts, metal nanoparticles, antibiotics, acids, chitosan derivatives, plant extracts, bacteriophages and photodynamic inactivation (PDI) have been suggested as an alternative to destroy biofilms. The choice of one method over the other depends mostly on the cost and its effectiveness (Simões *et al.*, 2009; Beloin *et al.*, 2014).

1.7.1. Chemical compounds

As biofilms become increasingly challenging in many different areas such as medicine, food and water treatment there has been an effort to find viable sources of anti-biofilm agents. Chemical compounds such as nitroxides seem to have anti-biofilm properties (Alexander *et al.*, 2015). Nitroxides are a wide group with antioxidant properties (Soule *et al.*, 2007). These compounds are easy to handle and of fast preparation, so their chemical and biological reactivity, solubility and affinity towards cells can be customized for the desired application (Soule *et al.*, 2007). Nitric oxide (NO), a signaling molecule, appears to prevent the formation of *P. aeruginosa* biofilms and affects their dispersal (Alexander *et al.*, 2015). An anti-biofilm compound produced by marine sponges of the family *Agelasidae*, was recently discovered (Stowe *et al.*, 2011). These sponges produce a family of alkaloids compounds - oroidins - that possess nitrogen-dense architectures characterized by the incorporation of one or more 2-aminoimidazole sub-units. Oroidin anti-biofouling activity against the Gram (-) marine α-proteobacterium *Rhodospirillum salexigens*, and anti-attachment activity against *V. vulnificus* has been documented (Stowe *et al.*, 2011).

1.7.3. Enzymes

Since the biofilm matrix is made of DNA, proteins, and extracellular polysaccharides, the disruption of the biofilm structure can be accomplished using enzymes that degrade particular biofilm components (Thallinger *et al.*, 2013). Some studies reported that in *Streptococcus pneumoniae,* DNase I induced biofilm degradation by 66.7%–95% and the average biofilm thickness was also reduced by 85%–97% (Taraszkiewicz *et al.*, 2012). Enzyme-based detergents are increasingly used in the food industry and have also been used as synergists to improve disinfectant efficacy. However,

it has been difficult to identify enzymes that are effective against all types of biofilms and the use of cocktails of several proteases and polysaccharide hydrolyzing enzymes has been suggested (Simões *et al.*, 2009).

1.7.4. Phages

The use of bacteriophages, has gained interest as a strategy for biofilm control once their abundance and ubiquity, make them realistic means of destroying biofilms (Skurnik and Strauch, 2006; Hanlon, 2007). Bacteriophages encode enzymes such as EPS depolymerases, endolysins, or simply lysins that are capable of degrading EPS (Chan and Abedon, 2015). Biofilm inactivation results from the combination of enzymatic EPS degradation lysis of embedded bacteria (Skurnik and Strauch, 2006; Hanlon, 2007; Chan and Abedon, 2015). The use of bacteriophages reduces alginate viscosity in *P. aeruginosa* biofilms and this effect seems to be related to the degradation of exopolysaccharides by enzymes produced by the bacterial host (Simões *et al.*, 2009). Although the infection process is affected by the chemical composition of the medium, temperature, growth stage of the cells and phage concentration (Simões *et al.*, 2009), there are still significant gaps in the understanding of the mechanisms of bacteriophage control of biofilms.

1.7.5. Quorum sensing quenchers/inhibitors

Quorum quenching (QQ) happens when some process or substance interferes with the normal functioning of quorum sensing and many strategies for disrupting a bacterial quorum sensing system and inhibiting biofilm formation have been discovered (Rasmussen and Givskov, 2006).

Quorum quenching enzymes are able to degrade or modify the signal molecule (e.g. AHL). There are two types of AHL degrading enzymes: the AHL-lactonase and AHL-acylase (Amara *et al.*, 2011; Worthington *et al.*, 2012). AHL-lactonase cleaves the ester bond of the lactone ring, resulting in N-acyl homoserine, and AHL-acylase cleaves the amide bond between the acyl chain and the homoserine lactone ring (Amara *et al.*, 2011) On the other hand, oxidoreductase modifies the AHL to 3-hydroxy AHL, resulting in hydrolyzed or modified componds that don´t function as signal molecules for biofilm formation (Uroz *et al.*, 2005).

Some bacteria are also capable of producing and excreting biosurfactants. It has been recently observed that *cis*-2-decenoic acid produced by *P. aeruginosa,* was able to induce the dispersion of established biofilms. This molecule has been successfully tested exogenously, against *B. subtilis, E. coli, Staphylococcus aureus, Klebsiella pneumoniae, P. mirabilis, Streptococcus pyogenes* and the yeast *Candida albicans* (Davies and Marques, 2008; Jennings *et al.*, 2012).

1.8. Photodynamic inactivation

Photodynamic inactivation (PDI) is a process that involves the combination of a nontoxic photosensitizer (PS), visible light and molecular oxygen to generate cytotoxic species that will attack vital cellular components and inactivate cells. This approach has been gaining interest as an alternative to chemical antimicrobial approaches since it has a broad spectrum of action, it is efficient in the inactivation of antibiotic-resistant strains, has low mutagenic potential and strains that are resistant to direct effects of light, such as UV radiation (Maisch, 2015).

PDI has been successfully tested against virus, bacterial cells, endospores, fungi, parasites and microbial biofilms (Jori *et al.*, 2006; Maisch, 2015). Antibiotic-resistant strains are as susceptible to PDI as sensitive wild-type strains and because PDI it is a multi-target process, resistance mechanisms are not likely to develop. Therefore, PDI is regarded as a promising approach for the inactivation of microbial biofilms, namely those related with human health or environmental deterioration.

1.8.1. The photodynamic effect

Photodynamic effect relies on the interaction of light with a light-absorbing molecule named photosensitizer (PS) that will further interact with molecular oxygen. The PS is excited, by absorbing a certain amount of energy from the light, to a long-lived triplet state, from which energy can be transferred to biomolecules or directly to molecular oxygen, depending on the reaction type **(Fig.1.4)**.

Photodynamic therapy (PDT) is normally used as a therapy for cancer and other non-infectious diseases (Dougherty *et al.*, 1998). When the cells to be killed are microorganisms the procedure is named photodynamic inactivation (PDI) and when it is

used in the medical context to treat infections, it is referred as photodynamic antimicrobial chemotherapy (PACT). PDI/PACT exhibit several positive aspects, once it has not as yet been possible to artificially induce resistance to PDI in any microbes where it has been tested (Maisch, 2015). Like PDT, PDI uses photosensitizers and ultraviolet or visible light to create a phototoxic response, normally via oxidative damage. The major use of PACT is in the disinfection of blood products, particularly for viral inactivation and in the treatment of oral infection (Wainwright, 1998).

The type I photodynamic mechanism involves the generation of reactive oxygen species (ROS) owing to interactions between the excited photosensitizer and a molecule in its immediate surrounding area via hydrogen abstraction or electron transfer. ROS are formed from electron transfer to molecular oxygen (Plaetzer *et al.*, 2009).

In type II photodynamic mechanism, the PS in the excited state will transfer energy to the triplet state molecular oxygen generating highly reactive singlet ($^1O_2$) that will oxidize biomolecules in the cell such as proteins, lipids and nucleic acid, ultimately leading to cellular damage and cell death. Both mechanisms can occur simultaneously in the cell, but type II mechanism is generally considered to be the major contributor to PDI of microbial cells with porphyrin PS (Tavares *et al.*, 2011; Costa *et al.*, 2013).

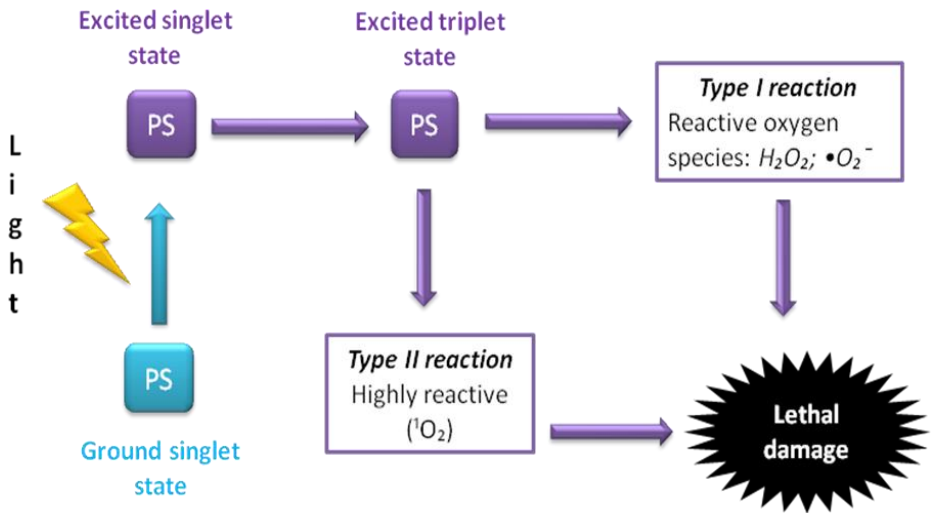


Fig.1.4 - Scheme of photodynamic inactivation processes (adapted from Singh *et al.*, 2014).

1.8.2. Photosensitizers

A suitable PS for PDI must be a chemically pure light-absorbing substance, able to produce singlet oxygen or other ROS, representing low mutagenic risk and negligible dark toxicity and displaying a broad spectrum of action against viruses, bacteria, fungi and parasites (Maisch, 2015).

The major types of PS can be classified as tetrapyrrole and non-tetrapyrrole compound **(Fig. 1.5)**, from synthetic or natural origins (Castano *et al.*, 2004). PS derived from aromatic tetrapyrrolic nucleus have a relatively large absorption band in the region of 400 nm (Castano *et al.*, 2004), and are the most common PS for PDI.

The first generation of photosensitizers is represented by hematoporphyrin and derivatives (HpD) such as the porphyrin-based Photofrin®, Photosan®, Photocan® (Allison *et al.*, 2004). The absorption spectrum of porphyrins **(Fig.1.5)** is characterized by a Soret band of in the 420–430 nm region (Almeida and Cunha, 2011), which makes them suitable for irradiation with blue light (Dai *et al.*, 2012) or natural sunlight (Costa *et al.*, 2010).

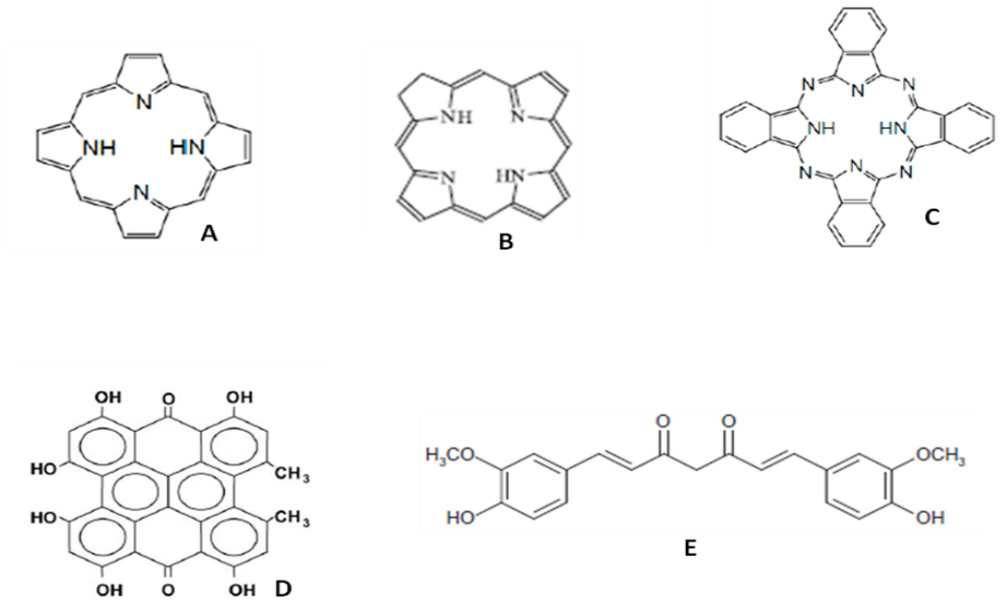
The second generation of photosensitizers such as benzoporphyrin, chlorins and phthalocyanines, which have a more intense long wavelength absorption, developed by modification of tetrapyrrolic (porphyrins) compounds. Nevertheless, most of these agents are still highly hydrophobic (MacDonald and Dougherty, 2001). Phthalocyanines have high molar absorption coefficient between 670 nm and 780 nm (Detty *et al.*, 2004), but are not easily soluble in aqueous media being usually prepared with sulfonic acid groups to provide a better water solubility (Castano *et al.*, 2004).

A third generation of photosensitizers was developed in the attempt of solving affinity and selectivity problems of earlier PS. Selective delivery of PS to the tumor tissue, was assured by conjugation with biomolecules, such as monoclonal antibodies. Another improvement in the PS efficiency may be given by the nanomaterials, once they demonstrate potential for the improvement of drug delivery to the target area, resulting in the maximum therapeutic efficacy (Paszko *et al.*, 2011). They may also improve the solubility, solving the hydrophobicity problem of some PS, minimize the degradation of the drug after being administrated, decrease side effects and enhance bioavailability

(Zhang *et al.*, 2008). Other presumable benefits may be the lower toxicity, better biocompatibility and safety (Paszko *et al.*, 2011).

Natural compounds like hypericin, curcumin and riboflavin, and synthetic dyes like methylene blue, toluidine blue O (TBO), which is partially soluble both in water and alcohol, and rose bengal, are good examples of non-tetrapyrrole PS that have also been successfully tested form the PDI of microorganisms **(Fig. 1.5)** (Zanin et al. 2006; Araújo et al. 2012; Cardoso et al. 2012; Cronin et al. 2012; Yow et al. 2012).

Curcumin is found in the rhizome of *Curcuma longa*. Commercial *C. longa* extracts contain also the curcuminoids demethoxycurcumin and bis-demethoxycurcumin (Dahl *et al.*, 1989). Curcumin is a crystalline compound with a bright orange-yellow color with a peak of absorbance at 418 nm, depending on the solvent (Lestari and Indrayanto, 2014) and it is used as dye and also as a food additive (E100). *In vitro* studies demonstrated that that curcumin has antimicrobial (Gunes *et al.*, 2013), antitumoral (Notarbartolo *et al.*, 2004), anti-inflammatory (Basnet and Skalko-basnet, 2011) and antioxidant properties (Asouri *et al.*, 2013).



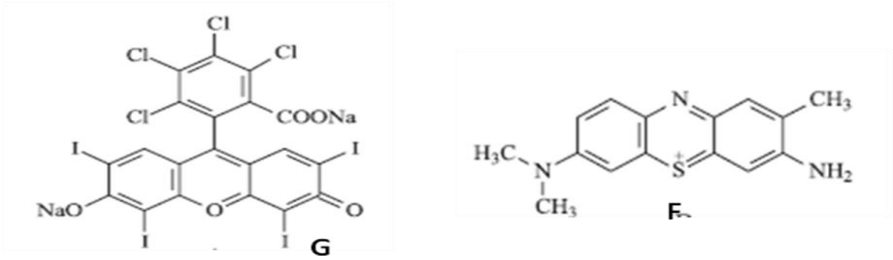


Fig. 1.5 - Chemical structure of photosensitizers used for the photodynamic inactivation of microorganisms. A - Porphyrins; B - Chlorins; C - Phthalocyanines; D - Hypericin; E - Curcumin; F - Toluidine Blue O; G - Rose Bengal.

1.8.3. Cellular and molecular targets in PDI of microorganisms

It is known that Gram (+) bacteria are much more susceptible to PDI, than Gram (-) bacteria (Perussi, 2007). This happens because of differences in the cell wall structure. Gram (-) bacteria are resistant to a large number of antimicrobial agents because they present an additional layer in the cell wall, the outer membrane, that is located externally to the peptidoglycan layer. The outer membrane is highly impermeable, showing an asymmetric structure composed of strong negatively charged lipopolysaccharides (LPS), phospholipids, lipoproteins and proteins with porin function, which excludes the penetration of several classes of molecules, diminishing the PDI effect (Pereira *et al.*, 2014). Differences in susceptibility to PDI between Gram (+) and Gram (-) will depend on a series of factors **(Table 3)** such as, membrane permeability barriers, differences in DNA antioxidant enzymes or DNA repair mechanisms as well as simple factors such as bacterial cell size. Features like hydrophobicity and charge of the PS, and affinity for the bacterial components are also important to an effective photoinactivation. For example, the bacterial membrane is also a primary target of photodynamic PDI, and phospholipids are attacked by ROS that cause a direct oxidative modification of unsaturated lipids and an indirect modification via reactive products of lipid peroxidation (Alves *et al.*, 2014).

Table 3| PDI action at molecular level (adapted from Wainwright and Crossley, 2003).

Site of action	Action	Result	Consequence	Antimicrobial event
Water	Hydrogen abstraction	Formation of hydroxyl radical (HO•)	Formation of H_2O_2, superoxide (O_2^-)	Oxidative process
Cell wall/Envelope: unsaturated lipids	Peroxidation	Peroxidation	Hydroperoxide formation	Increased ion permeability (Na^+/K^+ leakage)
Protein coat	Oxidation of Try/Met/ His residues	Protein degradation	–	Loss of viral infectivity
Viral protein coat	Hydrogen abstraction	Peptide cross-linking	Enzyme inactivation	Loss of repair facility
Nucleic acid residues	Oxidation of base or sugar	8-Hydroxy-guanosine formation	Nucleotide degradation; Sugar degradation/ cleavage	Base substitution strand cleavage, mutation, inhibition of replication
Enzymes (e.g. reverse transcriptase)	Oxidation/cross-linking	–	e.g. Inhibition of ribosome assembly	Inhibition of replication/ infectivity in viruses

Biofilms are usually more resistant to PDI than planktonic cells (Lin *et al.*, 2004; Beirão *et al.*, 2014). However, some studies have demonstrated that they are susceptible to PDI. (Li *et al.*, 2013), demonstrated a notable reduction in the number of adherent bacteria and the amount of extracellular matrix, followed by a light-dose-dependent disruption of the biofilm. Other studies have also demonstrated PDI of biofilms with only a few aggregated colonies left and single cells presenting large holes in the cell wall (Sbarra *et al.*, 2008; Eick *et al.*, 2013) According to these findings, the light and PS were able to penetrate the deepest layers of the biofilm.

1.9. Objectives

This work intended to assess the applicability of a food-compatible photosensitizer (curcumin) for the control of bacterial attachment to solid surfaces, represented by silicone tubing, and for the inactivation of installed biofilms, envisaging the application of PDI for the control of biofilms in food-contacting surfaces or packaging materials. An environmental strain of *Pseudomonas spp.* was used as a model biofilm forming microorganism. For comparative purposes, a porphyrin derivative already demonstrated as effective against a wide range of microorganisms was also tested.

2. Materials and Methods

2.1. Microorganism and growth conditions

Is this study, an environmental strain of the Gram negative bacterium *Pseudomonas spp.,* isolated from water of the surface microlayer of Ria de Aveiro (Louvado *et al.*, 2010), was used as model biofilm-forming microorganism. The choice of *Pseudomonas spp.* as biological model was based on the fact that it is a relevant microorganism in clinic and environmental contexts and biofilm formation, the composition of the matrix is well studied, and processes of quorum-sensing and relation with human health are well documented in the scientific literature (Kievit *et al.*, 2000). Stock-cultures in Tryptic Soy Agar (TSA, Merck) were refreshed weekly by streak-platting, incubated for 24h at 37 °C and stored at 4 °C. Before each assay, a fresh culture was prepared by inoculating an isolated colony from the stock-culture in 50 mL of Tryptic Soy Broth (TSB, Merck). The liquid culture was incubated at 37 °C with agitation at 170 rpm for 18 - 24h, until early stationary phase was reached (OD_{600} ~ 0,7 (≈10^7 CFU mL^{-1})).

2.2. PDI assays

The photodynamic inactivation assays were designed to evaluate the photodynamic effect on *Pseudomonas spp.* mature biofilms (eradication assays) and to assess the effect on the initial phase of cell adhesion (colonization assays). For comparative purposes, PDI of cell suspensions (planktonic cells) was also tested.

2.2.1. Photosensitizers

In this study, two photosensitizers were used: the tetracationic porphyrin 5,10,15,20-tetrakis(1-methylpiridinium-4-yl) porphyrin tetra-iodide (Tetra-Py^+-Me) **(Fig. 2.1)** and the natural dye 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione (curcumin) **(Fig.1.5 - E)**. Tetra-Py^+-Me is a porphyrin derivative widely used in PDI assays (Alves *et al.*, 2008, 2009). It was synthesized by the Organic Chemistry Group of the Chemistry Department of University of Aveiro and delivered in 500 µM stock-solution using DMSO as solvent. The stock-solution was stored in the dark, at room temperature, and sonicated for 15 min just before use, to assure total solubilisation of the porphyrin. A

1240 µM stock-solution of commercial curcumin (Acros, 99.8%) was prepared in acetone PA and stored at 4 °C until use.

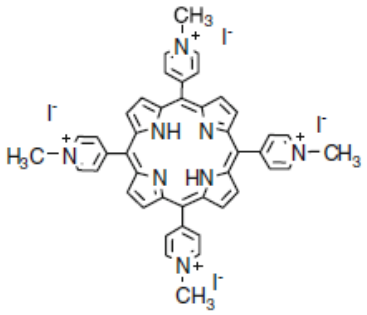


Fig.2.1 - Structural representation of the 5,10,15,20-tetrakis(1-methylpiridinium-4-yl) porphyrin tetra-iodide (Tetra-Py^{+}-Me) (Alves *et al.*, 2008).

2.2.2. Irradiation conditions

For PDI assays with cell suspensions and assays of inactivation of mature biofilms (eradication assays) a LumaCare® Lamp Model LC-122 equipped with fiber optic probes delivering white light (400-700 nm for assays with Tetra-Py^{+}-Me) or blue light (400 - 500 nm for assays with curcumin) was used. The irradiance was set to 150 mW cm^{-2} using a Power Meter Coherent FieldMaxII-Top combined with a Coherent PowerSens PS19Q energy sensor. Irradiation was conducted at room temperature for a maximum period of 30 min corresponding to a total light dose of 270 J cm^{-2}.

For colonization assays, a white light LED-setup (illumination platform) developed by the Photobiophysics Groups of the Physics Institute of the Humboldt University (Berlin) was used. The irradiance was set to 10 mW cm^{-2} and experiments were conducted at room temperature for a maximum period of 72h corresponding to a total light dose of 2592 J cm^{-2}.

2.2.3. Controls

In all PDI assays (cell suspensions, eradication and colonization assays), two control-conditions were included: dark controls (DC) consisted of biofilms or microbial suspension added of the maximum concentration of PS tested in each assay (50 µM for curcumin or 10 µM for Tetra-Py^{+}-Me) that were protected from light during the assays by

wrapping in aluminum foil; light controls (LC) were prepared and irradiated in the same way as tests but without the presence of PS.

2.2.4. PDI of planktonic cells

A fresh liquid culture of *Pseudomonas spp.* was ten-fold diluted in phosphate saline buffer (PBS; 1.44 g L^{-1} Na_2HPO_4, 0.2 g L^{-1} KCl, 0.24 g L^{-1} KH_2PO_4, 30 g L^{-1} NaCl, pH 7.4) and 1 mL aliquots were transferred to microtubes and added of 5 or 10 µM of Tetra-Py^+-Me or 5, 10 or 50 µM of curcumin. Samples were immediately protected from light with aluminum foil and incubated for 30 min in the dark, at 37 °C to allow the adsorption of the PS to bacterial cells. After the dark incubation period, microtubes corresponding to the light controls and tests were uncapped and irradiated as previously described. Dark controls were protected from light during the course of the experiments. At the beginning at the end of the irradiation, 100 µL aliquots were collected, pour-plated in duplicate in TSA. The cultures were incubated for 24 h at 37 °C. Colonies were enumerated in the plates corresponding to the most suitable dilution. The concentration of viable cells was calculated as the average colony counts corrected for the dilution factor and expressed as CFU mL^{-1}. Three independent assays were conducted for each condition and the results were averaged.

2.2.5. PDI of biofilms

2.2.5.1. Colonization assay

For the colonization assays, a fresh *Pseudomonas spp.* culture in TSB was prepared and ten-fold diluted in PBS. Aliquots of 3 mL were transferred to tissue culture flasks (30mL, Greiner Culture Flasks - Sigma Aldrich) and added of a stock-solution of porphyrin or curcumin to achieve a final concentration of 10 µM. Six silicone cylinders were added to each flask. The flask corresponding to the treatments and light control were placed on the illumination platform and irradiated for 24h (864 J cm^{-2}). The dark control was protected from the light with aluminum foil and subjected to the same temperature as the samples tested.

After the 24h-irradiation period, 3 silicone cylinders from each condition were collected, gently rinsed with PBS and individually transferred to microtubes containing 1

ml PBS and used for the determination of the concentration of viable cells in the biofilms, after the initial adsorption phase, as previously described.

The remaining silicone cylinders were also gently rinsed in PBS to remove loosely attached cells and transferred to a new flask containing 3 mL of 1/10 PBS and 10 µM of PS. The culture flasks were again placed on the illumination platform and irradiated form 48h (accumulated dose of 1728 J cm^{-2}) for biofilm maturation. At the end of the experiment, the silicone cylinders were collected, gently rinsed on PBS and individually transferred to microtubes containing 1 mL PBS. The concentration of viable cells was determined as described above for eradication assays.

2.2.5.2. Eradication assay

Biofilms were prepared in silicone cylinders as previously described, gently rinsed in PBS, individually transferred to microtubes containing 1 mL PBS and added of PS in order to achieve the final concentrations of PS in the aqueous suspension medium. Samples were protected from light with aluminum foil and incubated in the dark, at 37 °C for 30 min to allow for the adsorption of the PS to biological material. After dark incubation period, microtubes corresponding to the light controls and tests were uncapped and irradiated as previously described. Dark controls were protected from light during the course of the experiments. For the determination of the concentration of viable cells in the biofilms at the beginning and at the end of the experiment, sets of 3 microtubes containing the silicone cylinders were sonicated for 90 s and agitated in the vortex for 1 min in order to detach cells. The suspension was serially diluted (decimal dilutions) in PBS, and two replicates of the highest dilutions were pour-plated in TSA. The cultures were incubated for 24 h at 37 °C. Colonies were enumerated in the plates corresponding to the most suitable dilution. The concentration of viable cells was calculated as the average colony counts corrected for the dilution factor and expressed as CFU mL^{-1}.

2.2.6. Photosensitizer adsorption to bacterial cells

To determine the amount of PS attached to the biological material the procedure described in (Beirão *et al.*, 2014), was followed with adaptations related to the experimental conditions, microorganism and PS.

For the quantification of Tetra-Py^{+}-Me adsorption to planktonic cells, a microbial suspension was prepared (OD ≈10^{8} CFU mL^{-1}) and incubated for 30 min at 37°C with the same concentrations of PS as used in photodynamic inactivation assays (5 and 10 μM). Cells were collected by centrifugation (130 rpm, 15 min) and the supernatant containing unbound PS was discarded. The pellet was washed with 1 mL of PBS, the supernatant was discarded and the pellet was added of 1 mL of a digesting solution containing 0.1 $mol.L^{-1}$ NaOH and 1% sodium dodecyl sulfate (SDS) (Demidova and Hamblin, 2004). The samples were incubated in the dark at room temperature until clearance of the mixture or at least 24 h. For the quantification of curcumin adsorption, cells were digested with dimethyl sulfoxide (DMSO). For the quantification of the Tetra-Py^{+}-Me adsorption to biofilm cells, the procedure was similar to that used for the quantification of adsorption to planktonic cells, but mature biofilms were used instead of cells suspensions.

The concentration of each PS was determined by fluorescence (Spectrophotometer 3-Horiba Jobin-Yvon). The samples were excited in the region of the Soret band (420 nm), and emission registered within the range of 600-800 nm for Tetra-Py^{+}-Me and within the range of 425-750 nm for curcumin. The concentration of PS was calculated using a calibration curve previously constructed by adding known concentrations of PS to the corresponding digestion solution.

In parallel, the concentration of viable cells (CFU mL^{-1}) was quantified both in biofilms and in cell suspensions. For that, aliquots of the samples incubated in the dark, in presence of the PS, were serially diluted in PBS and plated in duplicate in TSA in order to count bacterial colonies. The amount of PS adsorbed to biological material was expressed in PS molecules CFU^{-1}. One assay, composed by 3 analytical replicates, was conducted for each experimental condition.

2.3. Statistic Analysis

The statistical analyses were performed with the two-way ANOVA test on Minitab 16 software in order to assess the statistical significance of the differences in the concentration of viable cells, between treatments. Analyses were performed with a significance level of 0.05.

3. Results

3.1. PDI of planktonic cells

The results of the assays of photodynamic inactivation of planktonic cells of *Pseudomonas spp.* using Tetra-Py^{+}-Me and curcumin are represented in **Fig.3.1 and 3.2**. Photodynamic inactivation with 5 µM of Tetra-Py^{+}-Me **(Fig.3.1)** caused a reduction of 3.7 log in the concentration of viable cells, after irradiation with a total light dose of 180 J cm^{-2}. With 10 µM, the inactivation factor was 3.0 log. There were no significant differences between the initial and final cell concentrations of both controls (DC and LC) (p=0.38).

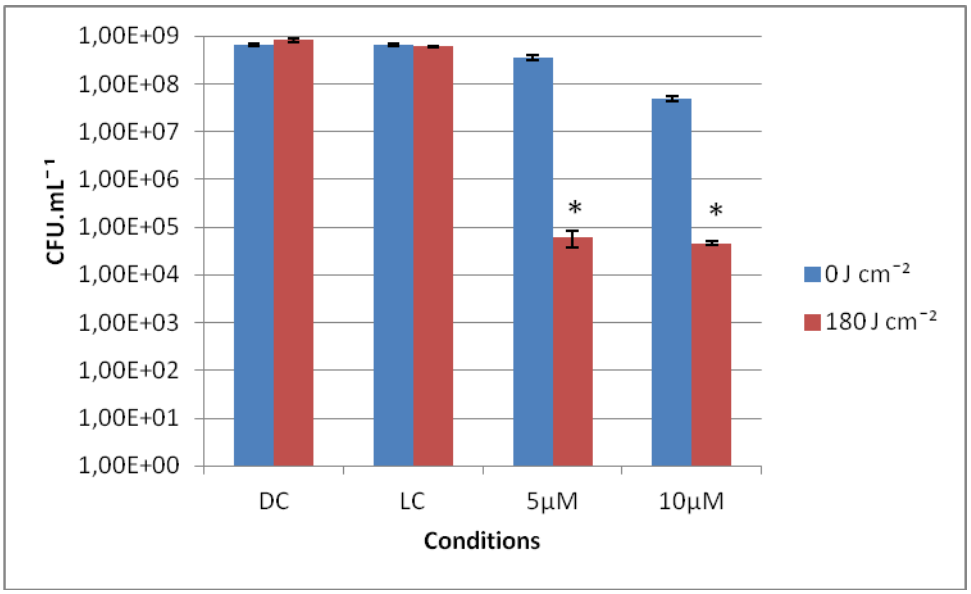


Fig.3.1 - Concentration of viable cells in suspensions of *Pseudomonas spp.* before and after irradiation with white light (400–700 nm) with a total energy dose of 180 J cm^{-2}, in presence of 5 µM or 10 µM of Tetra-Py^{+}-Me. * indicates significant differences between controls and the tests (5 µM and 10 µM) (ANOVA: $F_{2,18} = 339.99$, $p < 0.01$). LC=light control; DC=Dark control. Values represent the mean of three independent assays; error bars indicate the standard deviation.

Photodynamic inactivation with 5 µM of curcumin **(Fig.3.2)** caused a reduction of 0.7 log in the concentration of viable cells, after irradiation with a total light dose of 270 J cm^{-2}. With 10 µM, the inactivation factor was 0.9 log, and with 50 µM the inactivation factor was 0.7 log.

Similarly to what was observed in experiments with the cationic porphyrin, there were no significant differences between the initial and final cell concentrations of both controls (DC and LC), (p=0.91).

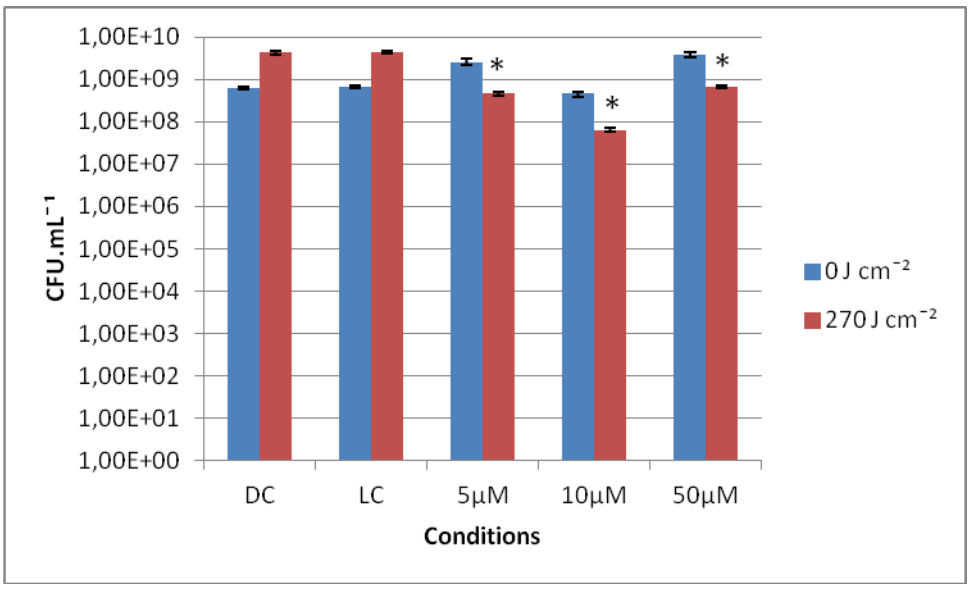


Fig.3.2 - Concentration of viable cells in suspensions of *Pseudomonas spp.* before and after irradiation with blue light (400–500 nm) with a total energy dose of 270 J cm^{-2}, in presence of 5 µM or 10 µM or 50 µM of curcumin. * indicates significant differences between controls and the tests (5 µM, 10 µM and 50 µM) (ANOVA: $F_{3,20} = 9.71$, $p < 0.01$). LC=light control; DC=Dark control. Values represent the mean of three independent assays; error bars indicate the standard deviation.

3.2. PDI of biofilms

3.2.1. Colonization assays

The results of the colonization assays are represented in **Fig.3.3 and 3.4**. In presence of 10 µM of Tetra-Py^{+}-Me **(Fig.3.3)** and with an accumulated light dose of 2592 J cm^{-2} (72 h), the final concentration of viable cells in biofilms was 5.2 log lower than the average concentration in the controls. During the period of adhesion (24 h), the accumulated light dose was 864 J cm^{-2}. In presence of the PS, a reduction of 2.2 log in the concentration of attached cells was observed, in comparison with the controls. During the maturation phase (48 h after transference of the solid support to new medium), a further reduction of 3.0 log occurred, in relation to the concentration of attached cells

determined at the end of the adhesion phase. In DC and LC the concentration of cells attached after the adhesion phase was not significantly different and there was a slight increase during the maturation phase (0.5 log in DC and 0.4 log in LC).

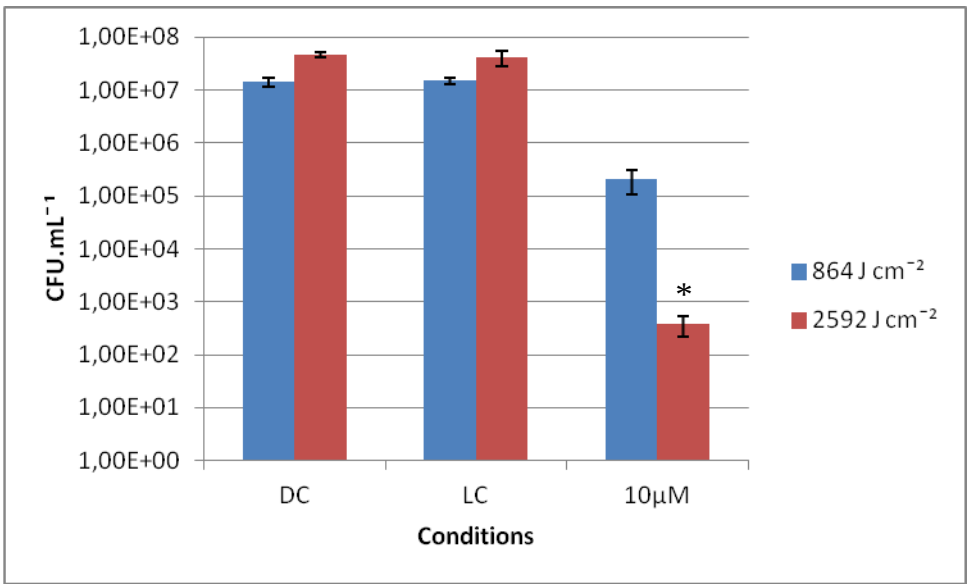


Fig.3.3 – Concentration of viable cells in biofilms of *Pseudomonas spp.* developing in presence of 10 µM of Tetra-Py^{+}-Me under irradiation with white light (10 mW cm^{-2}) after 24 h (864 J cm^{-2}) of incubation (adhesion phase) and further incubation after renovation of the culture medium (maturation phase) up to a total of 72 h (2592 J cm^{-2}). * indicates a significant differences between controls and the test (10 µM) (ANOVA: $F_{1,50}$ = 402.07, p <0.01). LC=light control; DC=Dark control. Values represent the mean of three independent assays and error bars represent the standard deviation.

The results of the colonization tests with curcumin **(Fig.3.4)** show that during adhesion phase (24 h; 864 J cm^{-2}), 10 µM of PS and light failed to cause a significant reduction (0.3 log) in the concentration of viable cells attached to the solid support. The extension of photosensitization throughout the maturation phase (further 48h; accumulated dose of 2592 J cm^{-2}) caused only a small reduction (1.0 log) in the concentration of viable cells in the biofilm, in relation to the average concentration in the controls. Similarly to what was observed in the colonization assays with the porphyrin, in DC and LC the concentration of cells attached after the adhesion phase was not significantly different and there was a slight increase during the maturation phase (0.7 log in DC and 0.8 log in LC).

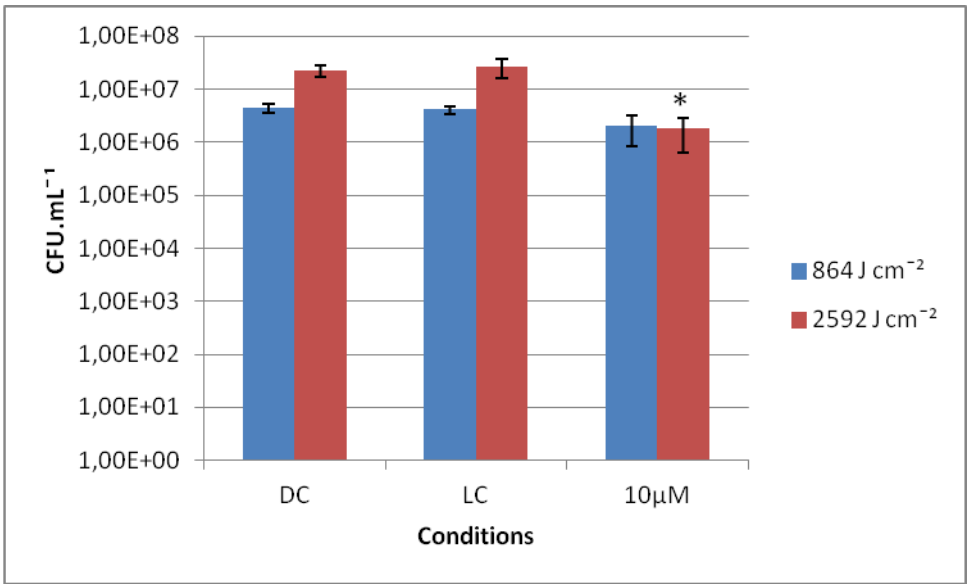


Fig.3.4 – Concentration of viable cells in biofilms of *Pseudomonas spp.* developing in presence of 10 µM of curcumin under irradiation with white light (10 mW cm^{-2}) after 24 h (864 J cm^{-2}) of incubation (adhesion phase) and further incubation after renovation of the culture medium (maturation phase) up to a total of 72 h (2592 J cm^{-2}). * indicates a significant differences between controls and the test (10 µM) (ANOVA: $F_{1,50} = 24.68$, $p < 0.01$). LC=light control; DC=Dark control. Values represent the mean of three independent assays and error bars represent the standard deviation.

3.2.2. Eradication assays

Considering the reduced effect of curcumin in planktonic cells, only the porphyrin Tetra-Py^{+}-Me was tested in the eradication assays. The results of the photodynamic inactivation of *Pseudomonas spp.* in mature biofilms are represented in **Fig.3.5.**

Photodynamic inactivation with 5 µM of Tetra-Py^{+}-Me **(Fig.3.5)** caused a reduction of 3.2 log in the concentration of viable cells, after irradiation with a total light dose of 180 J cm^{-2}. With 10 µM, the inactivation factor was 3.6 log. There were no significant differences between the initial and final cell concentrations in both controls (DC and LC) ($p=0.38$).

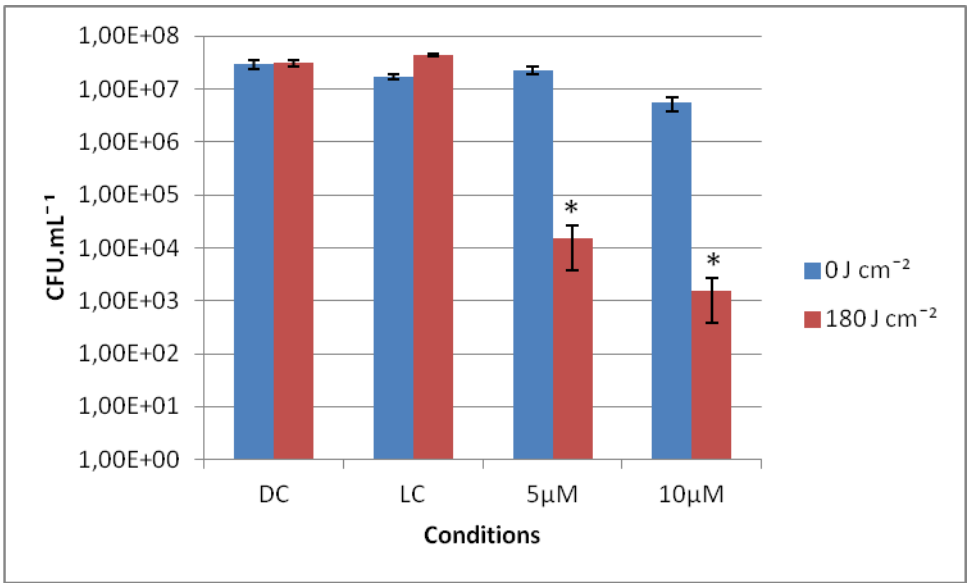


Fig.3.5 - Concentration of viable cells in biofilm of *Pseudomonas spp.* before and after irradiation with white light (400–700 nm) with a total energy dose of 180 J cm^{-2}, in presence of 5 µM or 10 µM of Tetra-Py^{+}-Me. * indicates significant differences between controls and the tests (5 µM and 10 µM) (ANOVA: $F_{2,18} = 339.99$, $p < 0.01$). LC=light control; DC=Dark control. Values represent the mean of three independent assays; error bars indicate the standard deviation.

3.3. Adsorption of the photosensitizers to bacterial cells

The results of the analysis of photosensitizer bound to biological material are represented in figure **Fig.3.6 and 3.7.** The adsorption of Tetra-Py^{+}-Me **(Fig.3.6)** to planktonic cells ($1.99*10^{8}$ PS molecules CFU^{-1} with 5 µM and $7.31*10^{7}$ PS molecules CFU^{-1} with 10 µM) was slightly higher than to biofilms ($1.34*10^{8}$ PS molecules CFU^{-1} with 5 µM and $4.84*10^{7}$ PS molecules CFU^{-1} with 10 µM). The adsorption of curcumin **(Fig.3.7)** was higher than that of the porphyrin and was also slightly higher in planktonic cells ($1.71*10^{9}$ PS molecules CFU^{-1} with 5.0 µM and $1.82*10^{10}$ PS molecules CFU^{-1} with 50 µM) than in biofilms ($5.75*10^{8}$ PS molecules CFU^{-1} with 5 µM and $2.19*10^{9}$ PS molecules CFU^{-1} with 50 µM).

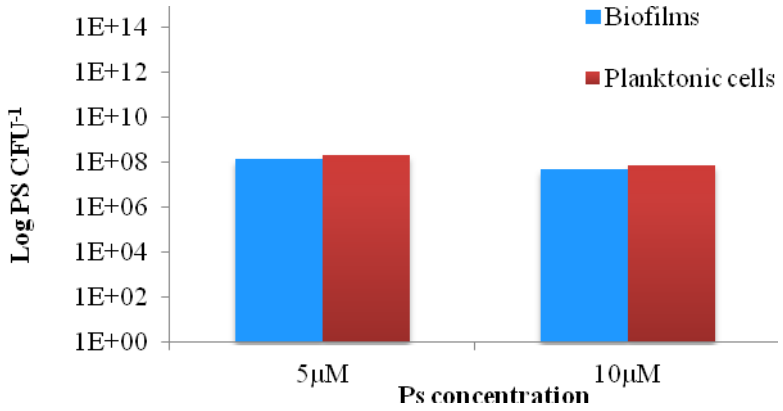


Fig.3.6- Amount of Tetra-Py^{+}-Me adsorbed to cells of *Pseudomonas spp.* in the planktonic and biofilm forms, when exposed to 5 µM and 10 µM during 30 min incubation in the dark, at 37 °C.

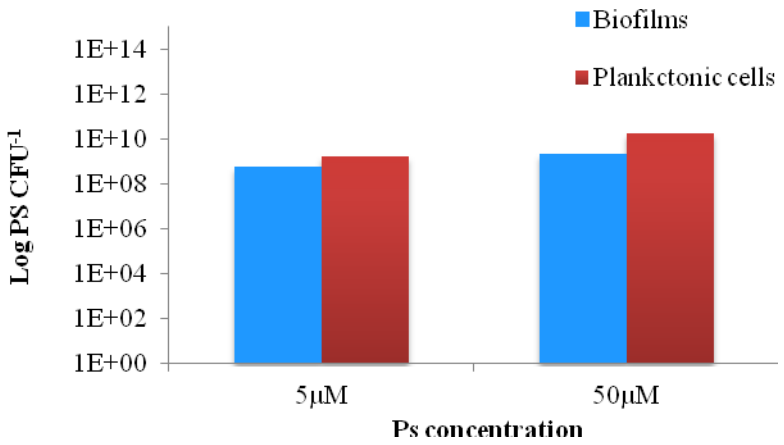


Fig.3.7- Amount of curcumin adsorbed to cells of *Pseudomonas spp.* in the planktonic and biofilm forms, when exposed to 5 µM and 50 µM during 30 min incubation in the dark, at 37 °C.

4. Discussion

Photodynamic inactivation of planktonic cells

The results obtained in this study confirm that the combination of a tetra-cationic porphyrin with white light represents a viable alternative for the inactivation of *Pseudomonas spp.* planktonic cells but, on the contrary, the combination of blue light with the natural photosensitizer curcumin had little effect.

The tetra-cationic porphyrin (Tetra-Py^{+}-Me) was tested as a reference PS in order to evaluate the susceptibility of the strain used in this work to the photodynamic process. Tetra-Py^{+}-Me has been extensively used for the PDI of bacteriophages and bacteria, even in the case of Gram (-). Several studies demonstrated the good inactivation capacity of the tetra-cationic porphyrin in Gram (-) bacteria. Reduction factors of 8.0 log for *Escherichia coli* with a concentration of 5 μM and a light dose of 64.8 J cm^{-2} (Alves *et al.*, 2009), 6.0 log for *Acinetobacter baumannii* with a concentration of 5 μM and a light dose of 64.8 J cm^{-2} (Almeida *et al.*, 2014) and 8.1 log for *Pseudomonas aeruginosa* with concentrations of 10 or 20 μM and a light dose of 43.2 J cm^{-2} (Almeida *et al.*, 2014) were reported. For *Pseudomonas aeruginosa,* a much lower inactivation factor (4.4 log) was attained with a higher energy dose (64.8 J cm^{-2}) and a lower Tetra-Py^{+}-Me concentration (5 μM) (Beirão *et al.*, 2014). As extensively demonstrated, Gram (-) bacteria, and particularly *P. aeruginosa,* are less susceptible to PDI that Gram (+) bacteria. Using a cationic porphyrin chloride (TriP[4]) as photosensitizer at a concentration of 12.5 μM, and a light dose of 27 J cm^{-2}, a reduction factor of 1.0 log was attained for *Pseudomonas aeruginosa* whereas under the same conditions, *S. aureus* and *C. albicans* showed 7.0 and 5.0 log decreases, respectively (Lambrechts *et al.*, 2005).

In this study, a maximum reduction of 3.7 log was obtained with 5 μM of Tetra-Py^{+}-Me and a light dose of 180 J cm^{-2}, and the inactivation efficiency did not improve when a higher concentration of PS (3.0 log with 10 μM) was tested. Although the strain used in this study is not the same used in the studies mentioned above, and significant differences in susceptibility between closely related bacterial strains have been reported (Grinholc *et al.*, 2008), the inactivation efficiency was in the range of the 4.4 log reported

by Beirão and co-workers (Beirão *et al.*, 2014) with another environmental isolate and the same PS concentration. In this study, white light with an intensity of 150 mW m^{-2} was used, instead of the PAR light with 4 mW cm^{-2} used by Beirão and coll. (Beirão *et al.*, 2014) and maximum inactivation of planktonic cells planktonic cells was achieved after 20 min irradiation, corresponding to an energy dose of 180 J cm^{-2} that is much higher than the dose (65 J cm^{-2}) used in the former experiments (Beirão *et al.*, 2014). Light spectrum, irradiance and irradiation time exert a significant influence on outcome of the photosensitization processes (Wainwright, 1998). It has been demonstrated that a lower irradiance rate applied for a longer period may be more efficient than a shorter treatment with a higher fluence rate (Costa *et al.*, 2010). This effect may explain the slightly lower inactivation factor obtain in this work when compared to that reported by (Beirão *et al.*, 2014) with a closely related bacterial strain and the same PS.

The use of curcumin as photosensitizer proved to quite ineffective in the inactivation of *Pseudomonas spp.* planktonic cells. To our knowledge, efficient photoinactivation of *Pseudomonas* in the planktonic form with curcumin has not yet been reported and it has been established that Gram (-) bacteria are less susceptible to PDI with curcumin that Gram (+) bacteria (Dahl *et al.*, 1989). Curcumin and light were successfully used for the reduction of the concentration of the Gram (+) bacteria *Streptococcus mutans* and *Lactobacillus acidophilus* in planktonic cultures (Araújo *et al.*, 2012). Efficient photosensitization (5.3 log inactivation) of *Streptococcus mutans* with 4 mM of curcumin and blue light (72 J cm^{-2} corresponding to 5 min irradiation at 240 $mW.cm^{-2}$) was also reported (Paschoal *et al.*, 2013). In the present study, the tested concentrations of curcumin (5, 10 and 50 µM) were much lower (80-800 times) than those used in the previously mentioned study. In concentrations higher than 200 µM curcumin exhibits antibacterial properties in the absence of light. Therefore, it is possible that high inactivation factors reported with mM concentration of curcumin activated by light may result from a dark-toxicity effect combined with a photosensitization process (Gunes *et al.*, 2013). In fact, curcumin may even exert a protective effect against oxidative stress. In *Escherichia coli* and *Bacillus megaterium*, curcumin showed antioxidant

properties that offer protection against DNA damage caused by light (Sharma *et al.*, 2000).

It is known that Gram (+) bacteria are generally more susceptible to PDI than Gram (-) bacteria. Gram (-) bacteria contain an additional outer membrane, external to the peptidoglycan layer, and shows an asymmetric lipid structure composed by strongly negatively charged lipopolysaccharides (LPS), lipoproteins and proteins. Successful photoinactivation of Gram (-) bacteria depends on the chemical structure of each PS and on its ability to penetrate the outer membrane in order to reach inner layers of the cell wall and ultimately, the cytoplasmic membrane (Perussi, 2007).

The poor solubility of curcumin in water at neutral pH due to strong hydrophobicity of the conjugated alkene chain, and the unavailability of a strong polar group making it insoluble in polar solvents may be another possible explanation (Jagannathan *et al.*, 2012). Since experiments of inactivation of planktonic cells were conducted in aqueous medium (PBS), some aggregation of the PS may have occurred.

The results of the light and dark controls indicate that none of the tested photosensitizers had a direct toxic effect, in the absence of light, and that the *Pseudomonas* sp. strain was not significantly affect by light alone.

Photodynamic inactivation of biofilms

Considering the low susceptibility of planktonic cells to PDI with curcumin and that biofilms are more resistant to physical and chemical stress than planktonic cells (Mah and O'Toole, 2001), curcumin was not tested for the eradication of installed biofilms. To our knowledge, efficient photodynamic inactivation of *Pseudomonas* biofilms with curcumin has not yet been reported. In fact, one single study of PDI of biofilms with curcumin activated with blue light demonstrated that, the exposure of *Streptococcus mutans and Lactobacillus acidophilus* biofilms to 2 mM, 4 mM and 8 mM of curcumin and subsequent irradiation (fluency of 5.7 J cm^{-2}, for 5 min) resulted in 97.5, 95, and 99.9 % reductions ($p < 0.05$) in the concentration of viable cells and a decrease of 100% occurred when the curcumin concentration was 11 mM and 14 mM (Araújo *et al.*, 2014). However, such high

inactivation factors were obtained with Gram (+) bacteria and very high concentrations of curcumin.

The tetra-cationic porphyrin (Tetra-Py^{+}-Me) was tested for the inactivation of installed biofilms of *Pseudomonas spp*, since it was effective in the inactivation of planktonic cells. Tetra-Py^{+}-Me had already been tested in biofilms of Pseudomonas and, in presence of the highest concentration of PS (20 μM), the maximum inactivation corresponded to a 2.8 log reduction in the concentration of viable cells. This corresponded to a plateau in the inactivation kinetic profile, observed after 90 min of irradiation (21.6 J cm^{-2}), after which higher light doses did not significantly increase biofilm inactivation (Beirão *et al.*, 2014). In the present study, 3.2 log and 3.6 log reductions were attained with 5 μM and 10 μM, respectively, and a light dose of 180 J cm^{-2} which corresponds to a slightly more efficient inactivation, probably because of intrinsic differences in the susceptibility of the biofilm-forming strains. Using a very high concentration (225 μM) of a cationic porphyrin on *P. aeruginosa* biofilms, a 4.1 log reduction was attained after irradiation with 220-240 J cm^{-2} (Collins *et al.*, 2010).

In the present work, the maximum inactivation of installed biofilms (3.6 log) was similar to that caused in planktonic cells (3.7 log) with equivalent concentrations of PS and light doses, indicating that biofilms of this particular *Pseudomonas* strain did not express enhanced resistance to PDI, as usually observed. Considering that normally, the cell arrangement in biofilms and the extracellular polymeric substances of the matrix provide protection against physical and chemical stress, this result is somewhat unexpected and again, probably related to particular features of this strain or to the chemical composition of the matrix.

The results of the colonization assays demonstrate that the tetra cationic porphyrin was able to reduce the attachment of cells to the silicone tubes and also inactivate attached cells during the period that would correspond to biofilm maturation. When cells were irradiated in the presence of Tetra-Py^{+}-Me during the adhesion phase (accumulated light dose of 864 J cm^{-2}) the concentration of attached cells was 2.2 log lower than in the controls and by extending the irradiation during the maturation phase (accumulated light dose of 2592 J cm^{-2}), the concentration of cells in treated biofilms was

3.0 log lower than at the end of the attachment phase and 5.2 log lower than the controls. The results indicate that both stages of biofilm development (attachment and maturation) were negatively affected by photosensitization but considering the significant reduction in the concentration of viable cells during the maturation, phase under irradiation and in presence of the photosensitizer, there is evidence for a stronger effect in biofilm maturation that on initial cell adhesion to the silicone substrate. Although in eradication assays the inactivation factor (3.6 log) was smaller than in the colonization assays (5.0 log), the total energy dose was approximately 15 times higher in the later. Therefore, the efficiency of the two processes cannot be directly compared.

The results of colonization experiments using curcumin as photosensitizer indicate that the prevention of biofilm development was less efficient than with the cationic porphyrin. Photosensitization with curcumin did not significantly affect cell attachment and had a small effect on biofilm maturation. At the end of the experiment, the concentration of cells in the photosensitized biofilm was only 1.0 log lower than in the controls. The poor susceptibility of *Pseudomonas spp.* biofilms to curcumin may be related to the high concentration of alginate found the extracellular matrix (Mann and Wozniak, 2012). Actually, some protein residues associated with alginate may affect the stability of curcumin. The photo degradation of curcumin by light in presence of alginate is more rapid in aqueous solution than in organic solvents due to intermolecular H-bond formation (Tønnesen, 2006). This degradation appears to occur when the non-bonding electrons on the oxygen atom of the OH-group in curcumin become engaged in intermolecular hydrogen bonding, which leads to an increase in destabilization of the excited state by an increase in hydrogen-bonding capacity of the aqueous medium (Tønnesen, 2006).

The adsorption of photosensitizers to bacterial cells indicates that the extracellular matrix of biofilms is permeable to PS that bound as efficiently as to planktonic cells. This was also reported and interpreted as an evidence that other factors, besides the amount of adsorbed PS, affect the efficiency of biofilm PDI (Beirão *et al.*, 2014). The chemical composition of the matrix and the quenching effect on ROS may me particularly relevant and impose a limitation on biofilm photosensitization.

5. Conclusion

This work intended to assess the applicability of a food-compatible photosensitizer (curcumin) for the control of *Pseudomonas spp.* biofilms installed on a solid surface (silicone cylinders) and also the effect of photosensitization on different phases of biofilm development (cell adhesion and biofilm maturation). The cationic porphyrin Tetra-Py^{+}-Me was included in the assays as a reference photosensitizer and the inactivation of *Pseudomonas spp* in the planktonic form was also performed for comparison.

The tetra-cationic porphyrin was more effective than curcumin in the inactivation of both planktonic cells and biofilms, and it also inhibited the initial phases of biofilm development reducing cell attachment and inactivating cells during the maturation phase. Therefore, PDI can be regarded as a promising strategy not only to eliminate installed biofilms but more importantly, to prevent their installation.

Curcumin demonstrated reduced efficiency against planktonic cells, no effect on cell attachment and only a small effect on biofilm maturation. This intrinsic resistance of *Pseudomonas spp*. photosensitization with curcumin may be explained by the chemical composition and structure of the cell was of this Gram (-) bacterium and also by the chemical composition of the extracellular matrix of biofilms.

PDI is regarded as a viable, affordable and effective strategy for the as control and removal of biofilms, that can rely on inexpensive energy sources and circumvent the natural resistance of biofilms to antimicrobial compounds. However, biofilms still represent a significant challenge and the design of efficient photosensitizers that can maintain stability and be efficiently adsorbed to the biofilm material is an important aim for future developments. The combination with matrix-destabilizing agents can be used to enhance the access of the photosensitizer to embedded cells.

The results demonstrate that photosensitization significant reduced cell adhesion to a solid surface and inhibited biofilm development. This represents a promising perspective for new photodynamic anti-fouling strategies.

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