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Multidrug resistant bacteria inactivation by photodynamic therapy

Inactivação de bactérias multirresistentes por terapia fotodinâmica

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Keywords Multi-drug resistant bacteria, antimicrobial photodynamic therapy, porphyrins, hospital residual water, antibiotics.

Abstract

The development of antimicrobials promoted the idea that diseases provoked by microorganisms would diminish and would be reduced to the insignificancy to human health. However, the great amount of antibiotics used in human medicine and veterinary lead to a selection of pathogenic bacteria resistant to multiple antibiotics, being hospital wastewaters one of the most important sources of antibiotic-resistant organisms and antibiotic-resistance genes that are released into the environment.

The significant increase in the development of multiple resistance mechanisms to antibiotics caused an increase in the research of alternative treatments that may be cost effective and human friendly. Antimicrobial photodynamic therapy (aPDT) is a quickly expanding technology for the treatment of diseases since it inactivates efficiently microorganisms, is cost effective and human safe.

The general objective of this work was to assess the inactivation of 4 clinical multidrug-resistant bacteria by aPDT, using a tetracationic porphyrin (PS). The efficacy of aPDT was assessed in phosphate buffered saline (PBS) and in hospital residual water for each isolated bacterium and for the bacteria mixtured all together. The synergistic effect of aPDT and antibiotics (ampicillin and chloramphenicol) was also evaluated as well as the effect of sodium dodecylsulphate (SDS) on aPDT efficiency.

The results show an efficient inactivation of multidrug-resistant bacteria in PBS using 5 µM of PS during 270 minutes in the presence of a light fluence rate of 40 $W.m^{-2}$ (reduction of 6 to 8 log). In the residual water, the inactivation of the 4 bacteria was also efficient and the decrease in bacterial number starts even sooner.

It was observed a faster decrease in bacterial number when aPDT was combined with the addition of ampicillin and chloramphenicol at concentrations of 16 and 32 µg mL^{-1} (MIC dose 32 µg mL^{-1} for both antibiotics). The efficiency of aPDT with a lower porphyrin concentration (2.5 µM) in the presence of antibiotics at MIC dose was not significantly different of that obtained when just the PS was used. The addition of SDS did not affect the efficiency of aPDT.

The results of this study showed that aPDT inactivate efficiently multidrug-resistant bacteria, in hospital residual water the bacterial inactivation is faster than in PBS, the combination of antibiotics and aPDT acts more efficiently than the aPDT alone, but aPDT in the presence of SDS does not affect the efficiency of bacterial inactivation.

In conclusion, aPDT is effective to combating microbial diseases transmitted by multidrug-resistant bacteria and can be used to increase the efficacy of classical antibiotics.

Palavras-chave Bactérias multi-resistentes, terapia fotodinâmica antimicrobiana, porfirinas, água residual hospitalar, antibióticos.

Resumo

O desenvolvimento de agentes antimicrobianos levou a pensar que as doenças provocadas por microrganismos diminuiriam, tornando-se insignificantes para a saúde humana. No entanto, a grande quantidade de antibióticos utilizados na medicina humana e veterinária levaram a uma selecção de bactérias patogénicas resistentes a muitos antibióticos, sendo os efluentes hospitalares uma das fontes mais importantes de organismos resistentes a antibióticos e de genes de resistência a antibióticos que são lançados no meio ambiente.

O aumento significativo no desenvolvimento de diversos mecanismos de resistência a antibióticos provocou um aumento na pesquisa de tratamentos alternativos que apresentem baixo custo e que não apresentem efeitos adversos para o homem. A terapia fotodinâmica antimicrobiana (aPDT) alternativa aos antibióticos para o tratamento de doenças, visto que inactiva eficientemente microrganismos, é barata e segura.

O objectivo geral deste trabalho foi avaliar a inactivação de quatro isolados clínicos de bactérias multirresistentes pela aPDT, utilizando uma porfirina tetracatiónica (PS). A eficácia da aPDT foi avaliada em solução tampão (PBS) e em águas residuais hospitalares para cada bactéria isolada e para a mistura das 4 bactérias juntas. O efeito sinergético da aPDT e antibióticos (ampicilina e cloranfenicol) também foi avaliado, assim como o efeito do dodecilsulfato de sódio (SDS) sobre a eficiência da aPDT.

Os resultados mostram uma inactivação eficiente de bactérias multirresistentes em PBS utilizando 5 µM de PS, durante 270 minutos na presença de 40 $W.m^{-2}$ de luz (redução de 6-8 log). Na água residual hospitalar, a inactivação das 4 bactérias foi igualmente eficiente, começado mesmo a diminuição do número de bactérias mais cedo que em PBS.

Foi observado uma redução mais acentuada no número de bactérias quando a aPDT foi combinada com a adição de ampicilina e cloranfenicol nas concentrações de 16 e 32 µg mL^{-1} (dose MIC de 32 µg mL^{-1} para ambos os antibióticos). A eficiência da aPDT com uma concentração inferior de PS (2.5 µM) na presença de antibióticos na dose MIC não foi significativamente diferente da obtida quando foi utilizado apenas a porfirina. A adição do SDS também não afectou a eficiência da aPDT.

Os resultados deste estudo mostraram que a aPDT inactiva bactérias multirresistentes de forma eficiente; em água de esgoto hospitalar a inactivação bacteriana é mais rápida do que em PBS, a combinação de antibióticos e aPDT actua de forma mais eficiente do que a APDT sozinha, mas eficiência da aPDT na presença de SDS não é afectada.

Em conclusão, aPDT é eficaz para combater doenças microbianas transmitidas por bactérias multi-resistentes e podem ser usados para aumentar a eficácia dos antibióticos clássicos.

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List of acronyms and abbreviations

µL	Microlitre
µM	Micromolar
µg	Microgram
A. baumannii	*Acinetobacter baumannii*
Amp	Ampicillin
ANOVA	Analysis of variance
aPDT	Antimicrobial Photodynamic Therapy
Ca^{2+}	Calcium ions
CFU	Colony forming units
Cm	Chloramphenicol
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
Gram (-)	Gram-negative
Gram (+)	Gram-positive
m	meter
Mg^{2+}	Magnesium ions
MIC	Minimal Inhibitory Concentration
mL	Millilitre
P. aeruginosa	*Pseudomonas aeruginosa*
PDT	Photodynamic therapy
PS	Photosensitizer
S. aureus	*Staphylococcus aureus*
Tetra-Py+-Me	5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide
TSA	Tryptic soy agar
TSB	Tryptic soy broth
W	Watt

1. Introduction

1.1. Hospital Wastewaters

Growth of cities, increase in population density, industrial development and the increase of garbage production per capita, turned hospital waste management into an important and multifaceted problem that requires efficient methodology and conformity with specific rules and regulations (Askarian et al., 2004a; Ortolan et al., 2007).

Hospital wastewaters are a big problem of public health care due to the huge amount of chemicals, pharmaceuticals and hormones released to the environment (Lin et al., 2005; Pauwels et al., 2006; Gautam, et al., 2007; Ortolan, et al., 2007). Some of those chemicals are genotoxic and suspected to be a possible cause of cancers observed in the last decades (Gautam et al., 2007; Ortolan et al., 2007). On the other hand, hospital wastewaters and farming facilities are the foremost source of pathogenic and antibiotic-resistant organisms and antibiotic-resistance genes that are released into the environment (Balcioğlu et al., 2002; Baquero et al., 2008). Since the effluents are discharged to the same extent as conventional urban discharge to the municipal sewage system without prior treatment (Darsy et al., 2002; Gautam et al., 2007; Boillot et al., 2008) and there is a frequent disposal of wastewater treatment plants to surface water, there is a widespread contamination of freshwater supplies with emerging contaminants (Lin et al., 2005; Sosiak et al., 2005; Pal et al., 2010).

1.1.1 Chemical characterization of hospital effluents

The volume of waste achieved in a hospital is conditioned by various aspects, such as number of beds, nature of health care administered, economical, social and cultural status of the patient and the prevailing condition of the area where the hospital is located (Askarian et al., 2004b). Nevertheless, the global physicochemical parameters of hospital effluents show them to be less or as pollutant than urban wastewaters (Boillot et al., 2008).

There is a myriad of hospital waste types. They can be divided in several categories, a main one similar to urban wastewaters and other ones specific of hospitals:

The residues of domestic nature include discharges resulting from the needs of individuals, like the releases from kitchens, cleaning detergents, laundry service or air-conditioning (Darsy et al., 2002; Askarian, et al., 2004a; Boillot, et al., 2008), and they have characteristics analogous to urban discharges (Askarian et al., 2004a; Tsakona et al., 2007).

The other classes of wastes specific to hospitals include disinfectants and antiseptics, active principles, heavy metals, radioelements (Darsy et al., 2002; Emmanuel et al., 2005; Gautam et al., 2007; Boillot et al., 2008), acids, alkalis, solvents, benzene, hydrocarbons and colorants (Boillot et al., 2008), insecticides, surfactants and endocrine disruptors, including hormones (Lin et al., 2005; Sosiak et al., 2005; Pal et al., 2010).

The most employed products for the disinfection of surfaces and medical material are chlorinated derivatives, aldehyde-containing products and betadine (Darsy et al., 2002). After application, some non-metabolized drugs are excreted by the patients (analgesics, antibiotics, anti-epileptics, β-blockers, hypocholesterolemics, anticancer-drugs, etc.), being a very important element for wastewater pollution (Darsy et al., 2002;Carballa et al., 2004; Emmanuel et al., 2005; Gautam et al., 2007; Boillot et al., 2008). Sometimes, unused drugs are also disposed into the drain (Emmanuel et al., 2005).

Some sort of therapies demand the use of toxic products, like haemodialysis that rejects not only toxins but also other chemical products from the disinfection unit; nuclear medicine service (both therapeutic and diagnostic) disposes radioactive elements that create solid and liquid residues that are susceptible of dispersion (Darsy et al., 2002; Boillot et al., 2008). As an example, iodine^{131} used to treat the hyperthyroidism of thyroid cancer can be released in patient urine (Boillot et al., 2008). The presence of high concentrations of adsorbable organic halogens (AOX) is linked to the presence of iodinated contrast agents used for radiotherapy, to certain drugs and their metabolites that may contain organohalogenic elements, to the use of

disinfectants and chlorinated solvents and to other substances from laboratorial use (Boillot et al., 2008).

Other chemicals such as Freon 113, glutaraldehyde, free chlorine, as well as alcohols, acetone, formaldehyde, acetaldehyde, ammoniums, phenols and several metals such as copper, lead, zinc and arsenic can also be found in wastewaters (Boillot et al., 2008).

Another example of a major group of contaminants is the surfactants group. These are amphiphilic compounds (compounds that contain both hydrophilic and lipophilic moieties) and their major functions include solubilisation, emulsification, dispersion, wetting, foaming, and detergent capacity, as well as antimicrobial activity in some cases. Biosurfactants are applied in detergents, paints, coatings, cosmetics and pharmaceutics (Xu et al., 2011).

1.1.2 Microbiological characterization of hospital effluents

Even though the presence of bacteria and viruses in hospital wastewaters is confirmed, these have generally lower values than those present in urban effluents (Darsy et al., 2002; Emmanuel et al., 2005). The low number of faecal bacteria detected in hospitals wastewaters or effluents is probably due to disinfectants and antibiotics (Emmanuel et al., 2005). Markers of water viral pollution like enterovirus and other viruses are also present in hospital wastewaters (Emmanuel et al., 2005; Gautam et al., 2007).

Some pathogens present in hospitals effluents may come from patient faeces and urine (Salmonelles, *Shigella* spp., coliforms, vibrions, streptococci, enterobactereaceae). Other bacteria (staphylococci, *Pseudomonas* spp.), viruses and parasites belong to the hospital environment and are susceptive of provoking nosocomial infections. In spite the fact that the overall amount of bacteria is lower in hospital wastewaters than in urban effluents (Emmanuel et al., 2005), some pathogenic bacteria such as *Pseudomonas aeruginosa* and pathogenic staphylococci have been found to be more concentrated in hospital waters (Darsy et al., 2002).

Hospital environment bacterial strains are characterized by its resistance to antibiotics (Darsy et al., 2002; Emmanuel et al., 2005) and one of the major risks is multiple drug resistance genes that these microorganisms might harbour, like vancomycin-resistant enterococci, methicillin-resistant *Staphylococcus aureus* and multidrug resistant pseudomonads, living in biofilms (sewage sludge flocs) (Rowan, 2011).

1.2. Pharmaceuticals and their relationship with drug resistance

Antimicrobials may be defined as chemotherapeutic agents that eradicate, inhibit or slow down the growth of microorganisms, and are extensively used for human and veterinary medicine, to boost the growth rate of animals utilized for food or in aquacultures to prevent diseases (Hirsch et al., 1999; Jones et al., 2005; Kümmerer, 2009a; Zhang et al., 2009; Rowan, 2011). Since its discovery by Fleming in the 20s, that extracted penicillin from a fungus belonging to the genus *Penicillium* (Elmolla et al., 2008), a huge variety of antibiotics was developed. Among the early compounds are sulfonamides, penicillin, and streptomycin. Soon after, came the tetracyclines, isoniazid, macrolides, glycopeptides, cephalosporins, nalidixic acid, and a variety of other molecular classes (Shlaes et al., 2004).

The side effects of these drugs are usually assessed for humans and animals, but the environmental impact of their manufacture and use is less well understood and just aroused interest a few years ago (Hirsch et al., 1999; Doll et al., 2003; Boxall 2004). The effects of some kinds of medicines are already known, like the anthelmintics used in veterinary medicine and antibacterial therapeutics (Boxall, 2004), but there is a huge amount of drugs that affects the environment, such as antibiotics, statins or cytotoxins used in cancer treatment (Andreozzi et al., 2003; Doll et al., 2003; Boxall, 2004). In many countries, high-use drugs like acetaminophen, acetylsalicylic acid, ibuprofen, naproxen (Boxall, 2004) and carbamazepine (Doll et al., 2003; Boxall, 2004) and the success of penicillin, streptomycin and tetracyclines made them widely spread in the environment (O'Riordan et al., 2005). Also, veterinary

medicine uses antibacterials, antifungals and parisiticides in aquaculture and agriculture that contribute to environmental stress (Boxall, 2004). Antibiotics used in clinical practice and animal husbandry are a big contributor to drug resistance and even a responsible hospital use of antibiotics have side effects, allowing the appearance of resistance in non-target bacteria (Salmon-Divon et al., 2004). Antibiotics use may also accelerate antibiotic resistance genes progress (Kümmerer, 2009a; Zhang et al., 2009).

Some of the effects that come from the use of drugs arise at low concentrations, even below the concentrations used in safety tests (Boxall, 2004). One problem that arises from the application of low concentrations of antimicrobials in the environment is the increase of antibiotic resistant bacteria, since the presence of antibiotics can produce a selective pressure that favours the organisms that possess genes coding for antibiotic resistance (Hirsch et al., 1999; Elmolla et al., 2008; Rahube et al., 2010).

By being inadequately degraded or removed in wastewater treatment plants (Sosiak et al., 2005), antibiotics may cause formation of toxic degradation products (Rowan, 2011). The concentration of these chemicals in the water is usually low (for example, $\mu g.L^{-1}$ for pharmaceuticals and $\mu g.L^{-1}$ in the case of estrogens). However, their biological effects can be dangerous to humans and aquatic life (Lin et al., 2005).

1.3. Drug resistant bacteria

The prodigious development of antimicrobials promoted the idea that diseases provoked by microorganisms would diminish and would be reduced to the insignificancy to human health. Nevertheless, resistance to antibiotics raised and this idea was refuted (Taylor et al., 2002; Jori et al., 2006a; Luksienė et al., 2009; Dai et al., 2010). This increase in the resistance mechanisms such as genes coding for antibiotic resistance (Hamblin et al., 2004; Elmolla et al., 2008) or plasmid mediated resistance (Kümmerer, 2009b) caused a boost in the research of alternative treatments (Taylor et al., 2002; Salmon-Divon et al., 2004; Lambrechts, 2005), that may be cost effective,

human friendly (Luksienė, 2009) and do not lead to development of resistance (Jori et al., 2006b)

1.3.1 Drug resistance in Gram-positive and Gram-negative bacteria

Even though it was long considered that the most problematic bacteria were Gram-negative, markedly in hospital setting, antibiotic resistance is a rising problem in Gram-positive bacteria as well they are isolated in both community and hospital-acquired infections (Barker, 1999).

The focal difference between Gram-positive and Gram-negative bacteria is in the cell wall. While Gram-positive bacteria have a dense peptidoglycan layer, composed of multiple individual layers of peptidoglycan enclosing cell membrane, Gram-negative bacteria have merely a thin layer of peptidoglycan surrounding cell membrane, which is then circumscribed by an additional outer membrane (Barker 1999; Albrecht et al., 2005; Jori et al., 2006a; Jori et al., 2006b) composed by lipolysaccharides and lipoproteins attached to peptidoglycan, and great outer membrane proteins designated porins (Barker, 1999; Parente, 2005). That fact makes them homogeneous but stratified (Parente, 2005). The outer membrane offers some extra protection from exogenous agents (Dahl et al., 1989; Poole, 2001; Hamblin et al., 2004; Parente, 2005; Jori et al., 2006b) and the low number of porins existing in it are fundamentally responsible for restricting the penetration of many substances, such as antibiotics (Barker 1999; Albrecht et al., 2005), and it is the reason for the difficulty in arranging photosensitizers that are effective against both types of bacteria (Albrecht et al., 2005)

1.3.2 Resistance Mechanisms in Bacteria

Resistance may be inherited or acquired by processes of genetic mutation or gene transfer. Some bacteria are inherently resistant to some classes of antibiotics or to one in particular, for instance *Pseudomonas* spp. is resistant to tetracyclines and to almost all kinds of penicillins, Enterobactereaceae are resistant to macrolides, all Gram negatives are resistant to glycopeptides and Gram positive bacteria to aztreonam

(Barker, 1999). This resistance may occur at the level of permeability of bacterium to the particular antibiotic or at the target site (Barker, 1999).

There is however, a large variety of mechanisms by which bacteria can enhance resistance to external threats, for example, thickening of outer wall, encoding new proteins preventing drug penetration, advent of mutants deficient on porin channels that would allow the influx of externally added chemicals (Jori et al., 2006a), enzymatic destruction or modification of the antibiotic, an increase in the efflux of the antibiotic from the cell, alteration or production of a new target site or over-expression of the drug target (Barker, 1999).

Important resistance structures in gram-negative bacteria are efflux systems, which are able to deviate various antimicrobial agents such as antibiotics, biocides, dyes or detergents. Merging this ability of excreting drugs with reduced drug entry by the membrane is a good protection from the harmful effects caused by antimicrobial agents (Poole, 2001). The efflux systems may be classified in five categories: major facilitator superfamily, ATP-binding cassette family, resistance nodulation family, small multidrug resistance family and multidrug and toxic compound extrusion family (Poole, 2001). Many multidrug efflux systems are associated with occurrences of multidrug/fluoroquinolone resistance. In terms of clinical aspects, the most important family is the resistance-nodulation-division (RND) family (Poole, 2001) that was initially attributed to gram-negative specifically, but now is known to be in all kinds of organisms (Tseng et al., 1999; Poole, 2001).

Gram-negative intrinsic and acquired resistance depends on restrained drug gathering and/or antimicrobial modification or even destruction. Since most gram-negative bacteria do not have a hydrophilic or, except in some cases, hydrophobic-uptake pathway and are highly resistant to hydrophobic antibiotics, another uptake pathway was proposed, the self-promoted pathway, in which polycationic antibiotics interact with a site on the outer membrane at which Mg^{2+} noncovalently cross-bridges adjacent lipopolysaccharide molecules (Hancock et al., 1984; Hancock et al., 1988). This outer membrane destabilization allows enhanced passage across the outer membrane of the chromogenic 1-lactam nitrocefin, protein lysozyme, and the hydrophobic fluorophores N-phenylnaphthylamine (NPN) and 1-anilino-8-

naphthosulfonate (Hancock et al., 1984). Therefore, Hancock et al., (1984) suggests that the uptake of polycationic antibiotic that cause the outer membrane disruption is also promoted. This theory is supported by the evidence that EDTA, a divalent cation chelator which removes Mg^{2+} and Ca^{2+} from outer membrane sites (Hamilton-Miller 1966; Hancock et al., 1984; Jori et al., 2006a), causes similar enhancement of uptake of lysozyme and β-lactams as well as enhanced killing by the polycationic antibiotics (Hancock et al., 1984). In addition, a single-point mutation of *Pseudomonas aeruginosa* makes the cells resistant to not only the polycationic antibiotics but also to EDTA, while external Mg^{2+} competes with both classes of agents (Hancock et al., 1984).

Resistant bacteria are transmitted from the environment to humans by direct or indirect contact; increasing evidences point to an association between clinical resistance and environmental resistance genes (Zhang et al., 2009).

1.4. Photodynamic Therapy

Photodynamic therapy (PDT) is a quickly expanding concept to the treatment of diseases, since it eliminates unwanted cells like cancer cells or infectious microbial cells (Jori et al., 2006b; Jori, 2006; Dai et al., 2009). PDT is most satisfactory to the treatment of localized infections, including the infections that become chronic after long-lasting chemotherapy (Jori et al., 2006b). There are many works where bacteria, yeast, fungi and viruses are inactivated by aPDT (Merchat et al., 1996; Orenstein et al., 1998; Embleton et al., 2002; Salmon-Divon et al., 2004; Demidova et al., 2005; Lambrechts et al., 2005; Jori et al., 2006a; Grinholc et al., 2007; Maish et al., 2007; Costa et al., 2008, Alves et al., 2009, Dai et al., 2009; Dai et al., 2010; Huang et al., 2010; Ragàs et al., 2010; Tavares et al., 2010).

1.4.1 History of PDT

The first reports of reactions induced by light came from Egyptians, Indians and Chinese, centuries ago (Valenzeno 1990; Grossweiner et al., 2005; Rodica, 2007) and they were used to treat an assortment of skins diseases like psoriasis, vitiligo, rickets, cancer and psychosis (Rodica, 2007) by the ingestion of plants containing natural photosensitizers like psolarens (Sternberg et al., 1998) or hypericin

(Wainwright, 1998) leading to the production of extremely reactive singlet oxygen. Despite that fact, the essence of PDT was only ascertained a century ago, when Raab found out that acridine orange killed paramecia, in the presence of light, even though both factors were not toxic by themselves (Valenzeno, 1990; Sternberg et al., 1998; Nestor et al., 2006). His mentor, Hermann von Tappeiner assigned the term "photodynamic" after several studies about this photooxidative process (Valenzeno, 1990; Szeimies et al., 2001) which is a light-activated biological process that requires the presence of molecular oxygen (Grossweiner et al., 2005). Shortly thereafter, von Tappeiner applied topical eosin and visible light to treat skin tumours, lupus vulgaris and condomilata lata (Nestor et al., 2006; Luksienė et al., 2009). However, the development of antimicrobial treatments based in photosensitization stopped soon after the advent of antibiotics, being used in the later decades mainly in tumour therapy (Castano et al., 2004; Hamblin et al., 2004; Luksienė et al., 2009). Of particular importance to the development of antimicrobial PDT (aPDT) was the emergence of antibiotic resistance (Wainwright, 1998; Hamblin et al., 2004; Dai et al., 2010) which required the development of alternative antibacterial therapies to overcome that problem (Salmon-Divon et al., 2004; O'Riordan et al., 2005). Occurrence of mutations in microorganisms, improper antibiotic prescription and not finishing the treatments, exacerbate the problem of resistance (Hamblin et al., 2004).

aPDT has the potential to be a highly efficient alternative for the treatment of multidrug resistant bacteria, allowing the disadvantages of antibiotics use to be surpassed.

1.4.2 Principles of PDT

PDT is based on the action of light of an appropriate wavelength and a PS, in the presence of molecular oxygen (Soukos et al., 1998; Kenoth et al., 2001; Castano et al., 2004; Hamblin et al., 2004; Demidova et al., 2005; Lambrechts et al., 2005; Rodica, 2007; Luksienė et al., 2009 ;Dai et al., 2009; Dai et al., 2010). In the presence of light, the PS in the ground state is excited to its triplet state, transferring the absorbed energy to molecular oxygen leading to the production of reactive oxygen species (ROS)

that are extremely toxic and able to oxidize the biological molecules (Demidova et al., 2005; Peng et al., 2007; Dai et al., 2009; Luksienė et al., 2009).

Two oxidative mechanisms of photoinactivation are implicated in the oxidation of the targets: Type I and Type II processes. In the first one, there is an electron-transfer from the PS in its triplet state to a substrate to produce radical ions. These will react with oxygen to produce cytotoxic species, such as superoxide, hydroxyl radicals and hydrogen peroxide, which remains in close proximity with the PS and the target (Castano et al., 2004; Jori et al., 2006b; O'Riordan et al., 2005; Wainwright, 1998; Nyman et al., 2004). Type II involves the PS triplet state, in which occurs energy transfer to ground state molecular oxygen with the subsequent production of excited singlet oxygen, which can oxidize many biological molecules like proteins, nucleic acids and lipids (Castano et al., 2004; Hamblin et al., 2004; Jori et al., 2006b; Luksienė et al., 2009; O'Riordan et al., 2005; Rodica 2007; Nyman et al., 2004).

Singlet oxygen is the most important ROS, involved in many environmental and health effects, along with the therapeutic effects of some drugs and photochemotherapy treatments (Dahl et al., 1989; Jori et al., 2006b). Singlet oxygen alone can kill bacteria, and this was proved after some studies where the PS was physically separated from the target, attributing to singlet oxygen alone the killing effect observed during PDT (Dahl et al., 1989; Hamblin et al., 2004; Jori et al., 2006b).

This therapy is as efficient in killing multi-drug resistant strains as native strains, microorganisms do not develop resistance to aPDT and its antimicrobial effect is faster than that of the usual antimicrobials (Hamblin et al., 2004; Lambrechts et al., 2005; Maish et al., 2007; Dai et al., 2010; Tavares et al., 2010; Costa et al., 2011)

1.4.3 Types of PS used in PDT

Most of the PS utilized in clinic, in the environment as well as in laboratory experiments, derive from tetrapyrrole aromatic nucleus (Fig. 1**Erro! A origem da referência não foi encontrada.**)(Castano et al., 2004; Nyman et al., 2005). Naturally occurring porphyrins (Castano et al., 2004; O'Riordan et al., 2005) are fully conjugated tetrapyrroles and vary in the number and type of side groups particularly carboxylic

acid groups (uroporphyrin has eight, coproporphyrin has four and protoporphyrin has two), while chlorins are tetrapyrroles with the double bond in one pyrrole ring reduced and bacteriochlorins have two pyrrole rings with reduced double bonds (Castano et al., 2004; Luksienė et al., 2009). Chemical derivatives from naturally occurring porphyrins and chlorins, like purpurins, pheophorbides, pyropheophorbides, pheophytins and phorbins also exist (Castano et al., 2004).

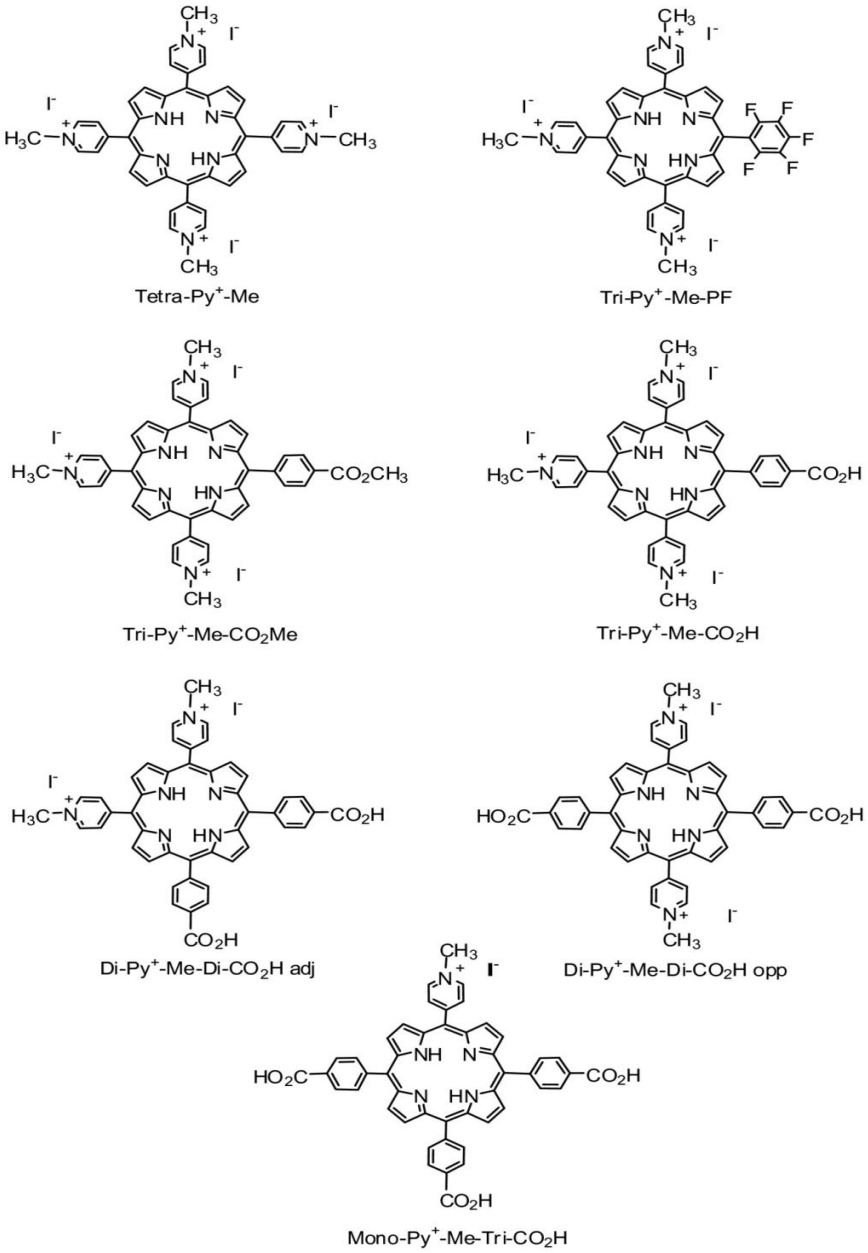

Fig. 1: Structure of the seven cationic porphyrin derivatives (Alves et al., 2009)

Other groups of tetrapyrrolic PS are phthalocyanines (Fig. 2) (Castano et al., 2004; O'Riordan et al., 2005) and naphthalocyanines. However, the four phenyl groups (or naphthyl) cause solubility and aggregation problems. Synthetic conjugated pyrrolic ring systems also used in aPDT includes texaphyrins, porphycenes and sapphyrins (Castano et al., 2004)

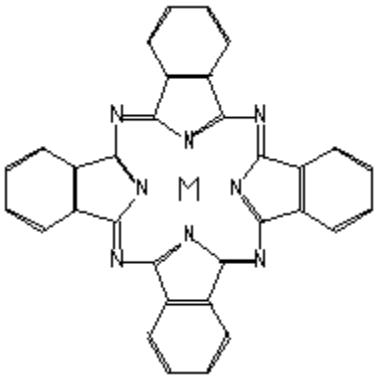

Fig. 2: Molecular structure of a typical metal(II) phthalocyanine

The PS more frequently tested in aPDT are based mainly in *meso*-tetraarylporphyrins (Almeida et al, 2011). The popularity of this type of PS results from their easy synthesis and potentiality toward further elaboration (Almeida et al, 2011). In fact, the synthetic approaches usually involve the condensation of pyrrole with adequate aldehydes, which are available in a wide range, providing porphyrins with different aryl or heteroaryl substituents at the *meso*-positions. Further manipulations of those substituents can give access to a high number of porphyrins that can be designed for the desired application (Almeida et al, 2011). The *meso*-tetra (N-methyl-pyridyl)porphine is a cationic porphyrin used to prevent infections by Gram-positive, Gram-negative or fungal pathogens. The advantage of this compound is the ability to attack a variety of cell components and low toxicity toward animal tissues at doses that can kill pathogens (Di Poto et al., 2009).

Some bacteria are also known to produce endogenous porphyrins (Luksiene, 2009). A few studies revealed that bacteria that produce reasonable amounts of endogenous porphyrins can be efficiently degraded by photosensitization, since there is no need of break through cell barriers (Luksiene, 2009).

1.4.4 Characteristics of photosensitizing agents (PS)

There are numerous studies that allude to diverse characteristics that a PS must have (Castano et al., 2004; Nyman et al., 2004; Jori et al., 2006a; Jori, et al., 2006b; Rodica, 2007; Luksienė et al., 2009). Overall, an ideal PS must:

- Be chemically pure,
- Produce singlet oxygen or other oxygen species,
- Be capable of accumulate in a microorganism in a strategic location,
- Use low cost sources for activation
- Be easy to deliver into the specific infection site
- Be quickly excreted
- Be a highly efficient killer, incorporating into vicious cells more efficiently than into normal cells
- Not be mutagenic or genotoxic
- Not be toxic in the absence of light,
- Have a broad spectrum of action, acting on bacteria, viruses, fungi, yeasts and parasitic protozoa,
- Be independent of the antibiotic resistance pattern
- Have a mechanism of inactivation that minimizes the risk of inducing the selection of resistant strains

The selectivity of the PS can be obtained by appropriate chemical design of the PS, which ensures that it will bind preferentially to microbial cells instead of mammalian cells (Dai et al., 2009).

Another important characteristic during the design of a PS for aPDT is their water solubility and positive charge, the latter being of utmost importance in gram-negative bacteria since their membrane structure excludes many anionic and uncharged lipophilic molecules (Dai et al., 2009), that would lead to phototoxicity in gram-positive bacteria (Hamblin et al., 2004). One way to bypass this problem is using a positively charged compound (Hamblin, 2004), usually based on tetrapyrrole nucleus (Luksienė, 2009) like phthalocianines, porphyrins (Jori et al., 2006a; Luksienė, 2009), chlorines, bacteriochlorines and texaphyrins (Luksienė, 2009). Porphyrins can be transformed to its cationic form by inserting a positively charged substituent in the

tetrapyrrole macrocycle (Jori et al., 2006a). These can promote photoinactivation of both gram-positive and gram-negative bacteria since gram-negative bacteria use the self-promoted uptake pathway to take up the photosensitizer (Hancock, 1988; Jori et al., 2006a).

In general, neutral or anionic PS are efficient to inactivate gram-positive bacteria but do not inactivate gram-negative bacteria after illumination like, for example, *Pseudomonas aeruginosa* or *Escherichia coli* (Hamblin, 2004; Salmon-Divon et al., 2004; Luksienė et al., 2009; Huang, 2010). This fact is explained by the differences observed on membrane physiology of both bacteria. As Gram-negative membrane is more differentiated and complex than in gram-positive bacteria , the photosensitizer penetrates through the membrane of Gram-positive bacteria and accumulate in the cell, while in Gram-negative singlet oxygen has to pass through the outer protection so it can reach the membrane or cytoplasmic components (Dahl, 1989). As the cell wall is the main difference between gram-positive and gram-negative bacteria, once this barrier is passed through, the mechanism of inactivation is identical (Dahl, 1989).

When cells are irradiated, formation of ROS starts degrading the cell wall (Luksiene et al., 2009). These ROS can interact with proteins, lipids, amino acid residues and nucleic acid bases as guanine and thymidine (Castano et al., 2004; Demidova et al., 2005; Luksienė et al., 2009).

1.4.5 Antimicrobial PDT (aPDT) and its application

aPDT is the application of PDT to treat infectious diseases of microbial origin (Jori et al., 2006b). aPDT relies on the accumulated experience in the treatment of malignant tumors by PDT. However, as the delivery of light is a localized process, this biomedical application is restricted to localized infections and not to systemic infections, such as sepsis (Hamblin et al., 2004; Demidova et al., 2005; Almeida et al., 2011). Contrarily to PDT for cancer, where the PS is usually injected into the bloodstream and accumulates preferentially in the tumour cells, aPDT for localized

infections is mostly carried out by local delivery of the PS to the infected area (Hamblin et al., 2004; Almeida et al., 2011).

aPDT has been applied not only in the clinical area but also in the environmental field (Jemli et al., 2002; Almeida et al., 2011). Although only a few studies have been conducted in this area, preliminary results suggest that aPDT has a great potential for environmental application, namely for use in water disinfection in treatment plants (DeRosa et al., 2002; Jemli et al., 2002; Carvalho et al., 2007; Costa et al., 2008; Alves et al., 2009) and in fish-farming plants (Almeida et al., 2011; Arrojado et al., 2011).

aPDT is considered a promising alternative to other kinds of treatment for several reasons, which include its broad spectrum of action, an efficacy independent of antibiotic resistance patterns, extensive pathogen reduction with limited damage to host tissue, specific delivery of PS in the infected area (Jori et al., 2006a; Jori et al., 2006b), low cost light sources, inexistence of photoresistant strains after multiple treatments (Jori et al., 2006b; Luksienė, 2009; Costa et al., 2011), absence of microorganisms viability recovery after treatment (Jori et al., 2006b; Tavares et al., 2010; Costa et al., 2011) and fast inactivation than usual antimicrobials (Hamblin et al., 2004; Lambrechts et al., 2005; Maish et al., 2007; Dai et al., 2010; Tavares et al., 2010).

In a clinical perspective, it can be established a group of factors that optimize the treatment of microbial infections by aPDT, allowing the selective killing of pathogens but not human cells. Those can be short incubation period, low PS concentration and soft irradiation (Jori et al., 2006).

The main targets of aPDT are the external microbial structures, as cell walls, cell membranes and nucleic acids (Käsermann et al., 1997).The damages to the external microbial structures can involve leakage of cellular contents or inactivation of membrane transport systems and enzymes (Gábor et al., 2001). Some damages produced in the nucleic acid chain can be repaired by the action of DNA repairing systems (Vzorov et al., 2002). It has been concluded that although nucleic acids damage occurs, it cannot be the principal cause of microbial photodynamic inactivation (Egyeki et al., 2003).

As the main targets of aPDT are the external structures, the PS does not need to enter in the microorganism. So, target microorganisms have no chance to develop resistance (Hamblin et al., 2004).

In an environmental perspective, some other aspects less relevant to aPDT in the clinic area, need to be taken into consideration, namely the removal of the sensitizer after photodynamic action to avoid the release of the PS to the water output; the determination of the stability of the PS conjugates under sun light irradiation conditions; the assessment of the impact of this procedure on the natural non-pathogenic microbial community structure; the toxicity of the PS on superior organisms at doses which induce marked mortality of microbial pathogens (Almeida et al, 2011).

1.4.6 Synergistic effect between aPDT and antibiotics

It is well known the use of large amounts of antibiotic compounds in clinical practice is undesirable, since they adversely affect the patient's condition and give rise to the selection of antibiotic-resistant strains, which consecutively increase the need of new and more powerful antibacterials. Therefore, being able to increase the efficacy of antimicrobial compounds, without administering them in large dosages has become an important aim of present-day research. However, little effort has been made to employ porphyrin derivatives in order to increase the efficacy of such antibiotics, reducing the dosages of these compounds needed to combat microbial infections.

Xing et al. (2011) tested a divalent vancomycin-porphyrin to inactive vancomycin-resistant enterococci (VRE). The divalent vancomycin-porphyrin showed strong aPDT activity against vancomycin-sensitive and resistant strains, when compared to vancomycin and porphyrin alone, with exceptional advantages necessary for multivalent interactions with the VRE and selective adhesion to bacteria cells leading to enhanced photodynamic inactivation and the divalent compound acts as a fluorescent probe to label and monitor bacterial strains effectively (Xing et al., 2011).

The combination of vancomycin with PDT was also useful in the disruption of *S. aureus* biofilms. Pre-treating the biofilms with PDT and then apply vancomycin at

concentrations bellow the biofilm inhibitory concentration, causes a disintegration of the biofilm matrix and allows the killing of bacteria almost entirely (Di Poto et al., 2009). Malik and Nitzan (1995) tested also the combination of different porphyrins (deuteroporphyrin and hemin) and antibiotics (methicillin, ampicillin, polymyxin B nonapeptide, tetracycline) to inactivate both Gram-positive (multi-resistant *S. aureus*) and Gram-negative bacteria (multi-resistant *E. coli*). The results of the study showed that in the presence of porphyrins and antibiotics the bacterial inactivation was higher than when porphyrins were used alone.

These results suggest that the aPDT effect in hospital wastewaters can be improved since the hospitals effluents have high amounts of antibiotics (Kümmerer, 2001)

1.5. Objectives

The general objective of this work was to assess the inactivation of clinical multidrug-resistant bacteria by aPDT, using a tetracationic porphyrin.

To reach this objective, the photoinactivation of the bacteria was studied in different conditions:

- using cultures of one bacteria and mixtures of four different bacteria
- in a known saline buffer solution (PBS)
- in samples of a hospital sewage water
- in combination with antibiotics
- in combination with SDS

2. Materials and Methods

2.1 Biological material

In this study it was used four multidrug-resistant bacterial strains [resistant to at least three from four common and representative drugs, for example, ceftazidime, imipenem, ciprofloxacin, tobrazidime (Jung et al., 2004)] with vital clinical importance: *S. aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Escherichia coli*.

Staphylococcus aureus is a gram positive emergent resistant bacteria to antibiotics that is regarded within staphylococci as the most virulent species due to its great heterogeneity of virulence factors, and its ability of forming biofilms, a fundamental factor in persistent infections caused by staphylococci (Di Poto et al., 2009). The biofilms can develop on central venous catheters or implanted medical devices and biofilm-associated infections can only be undertaken by removal of the infected piece (Di Poto et al., 2009). This bacterium is resistant to diverse antibiotics, namely to methicillin, considered one of the most effective antibiotics (MRSA) (Dai et al., 2010; Embleton et al., 2002; Jori et al., 2006a) and recently to glycopeptides antimicrobials, as vancomycin and teicoplanin (Embleton et al., 2002). They cause superficial and deep-seated skin, wound and tissue infections (Barker, 1999) and have turned into one of the predominant nosocomial pathogens colonising people for many months which calls for long hospital stays (Maisch et al., 2007). In some cases of MRSA skin infections may occur significant morbidity, or even life threatening situations (Dai et al. 2010), being the second major cause of bacteraemia, right after *E. coli*.

Pseudomonas aeruginosa is a gram-negative opportunistic pathogen, infecting immune compromised patients in hospitals (Masuda et al., 1995; O'Toole et al., 1998; Olayinka, 2004). Infections provoked by *Pseudomonas aeruginosa* are very often life threatening and not easy to treat. Although it can infect a diverse amount of tissues, they are more often founded in respiratory tract (O'Toole et al., 1998; Jung et al., 2004). This organism membrane has a low permeability level (Masuda et al., 1995), which takes native strains to have already limited susceptibility leading to a high

regularity emergency of resistance to drugs, especially with the regular use of broad-spectrum antibiotics (Jung et al., 2004).

Biofilm-grown *P. aeruginosa* has also been shown to acquire increased resistance to antibiotics (O'Toole et al., 1998; Mah et al., 2003), up to 1,000-fold greater than planktonic cells, whereas bacteria within these microbial communities employ distinct mechanisms to resist the action of antimicrobial agents (Mah et al., 2003).

Acinetobacter baumannii is a Gram-negative bacterium that captivated much attention due to its outstanding ability to acquire resistance to drugs and is becoming a growing problem in health systems, especially in burn infections units (Dai et al., 2009; Cisneros et al., 1996). It have been implicated in a variety of nosocomial infections, including bacteraemia, urinary tract infection, and secondary meningitis, but its main role at nosocomial pneumonia, particularly ventilator-associated pneumonia in patients confined to hospital intensive care units. Multiresistant *Acinetobacter* spp. is likely to be selected in the hospital environment in response to increasing antibiotic pressure (Bergogne-Bérézin et al., 1996).

Escherichia coli is a non-fermentative Enterobactereaceae pathogen in the community (Barker, 1999) and in hospitals, in particular in immunocompromised patients, being a great cause for diseases such as bacteraemia, pneumonia, skin and soft tissue infection and catheter-related sepsis (Barker, 1999) and its clinical management is complex because of the increasing incidence of infections caused by multidrug resistant strains (Manges et al., 2001).

Extended-spectrum β-lactamases are plasmid-mediated enzymes that confer resistance to oxyimino- β-lactams such as cefotaxime, ceftazidime, and aztreonam, antibiotics that were intended to be effective against strains producing known plasmid-determined β-lactamases (Jacoby et al., 1991).

Resistance to ampicillin and amoxicillin in *E. coli* is predominantly caused by the plasmid-encoded β-lactamase TEM-1, which is sensitive to β-lactamase inhibitors such as clavulanic acid. There are several mechanisms by which *E. coli* can be resistant to β-lactam–β-lactamase inhibitor combinations, such as amoxicillin plus clavulinic

acid. Since chromosomally encoded Bush group 1 β-lactamases are less sensitive than group 2 enzymes to inhibitors, overproduction of the *E. coli* chromosomal β-lactamase is one cause of this resistance. Some plasmid-encoded β-lactamases such as OXA-1 are less sensitive than TEM-1 to inhibition by clavulanic acid, so organisms that produce these enzymes are more frequently resistant to amoxicillin-clavulanic acid. Overproduction of TEM-1 also results in resistance, and also the deficiency in the OmpF and/or OmpC porins in conjunction with TEM-1 production. The most recently discovered mechanism of resistance to amoxicillin-clavulanic acid is production of β-lactamases derived from TEM-1 but with substantially reduced sensitivity to clavulanic acid and other β-lactamase inhibitors (Stapleton et al., 1995).

The bacterial strains were isolated from patients from University of Coimbra Hospital, (*Hospitais da Universidade de Coimbra - HUC*). *E. coli* was isolated from a sample of urine, *S. aureus* and *A. baumannii* were isolated from non-cirurgical wound exudates and *P. Aeruginosa* from expectoration samples.

The bacteria were identified and its profile of antibiotic susceptibility was characterized by the system BioMerieux Vitek 2® (BioMerieux).

Bacteria stocks were kept at -20ºC, in 40% glycerol and daily use bacteria were kept at 4ºC on tryptic soy agar (TSA) medium plates.

Before each assay, an aliquot of the selected bacteria was aseptically transferred to tryptic soy broth (TSB) and kept at 37ºC. Each bacterium was peaked up two more times before use.

2.2 Photosenstitizer

The PS applied in this study was a tetracationic porphyrin, the 5,10,15,20-tetrakis(1-methylpiridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^{+}-Me). The PS was prepared in two steps. First, the neutral porphyrin was obtained from the Rothemund and crossed Rothemund reactions using pyrrole and the appropriate aldehyde at reflux in acetic acid and nitrobenzene. After being separated by column chromatography (silica), the pyridyl groups were quaternized by reaction with methyl iodide (Alves et al., 2009).

A stock solution of this porphyrin, at 500µM, was prepared in DMSO and stored in the dark. Before each assay the porphyrin solution was sonicated during 30 minutes at room temperature. According to previous data (Alves et al., 2009), the PS concentration used in these assays was 5 µM.

2.3 Irradiation Conditions

It was used a set of white light lamps (PAR radiation, 13 lamps OSRAM 21 of 18W each lamp, with wavelength between 380-700nm) with a fluence rate of 40 $W.m^{-2}$ (radiometer LI-COR Model LI-250) as light source.

2.4 Photoinactivation Assays

2.4.1 Assays in PBS

Each isolate and a mixture of the four bacteria were tested in PBS. For the assays with the mixture of bacteria, each bacterium was grown separately and they were mixed just before the assay. It was used the same concentration of each bacterium.

For each assay three goblets were prepared, one with bacteria diluted 1:10 in PBS and 5 µM of PS (test microcosm); other with bacteria diluted 1:10 in PBS and 5µM of PS, which was kept in dark conditions, wrapped in aluminium foils (dark control) and other just with bacteria diluted 1:10 in PBS (light control). The goblets were kept under irradiation 270 minutes and aliquots were taken at 0, 30, 60, 90, 180 and 270 minutes after incubation. The aliquots were diluted in PBS and plated in TSA by incorporation. After 24h at 37ºC, the number of colonies was enumerated and the results were expressed in colony forming units per mL ($CFU.mL^{-1}$). All the assays were done in duplicate and for each assay two replicates were done.

2.4.2 Assays in hospital sewage water

The samples utilized in these assays were collected in the HUC at the days 10, 24 and 30 of November 2010, approximately at the same hour, 10h a.m.. The samples were transported to the laboratory and refrigerated at 4˚C until being utilized. The sewage water was filtered sequentially by 0,7 µm and by a 0,22 µm pore membranes, to eliminate bacteria and suspended organic matter of the water. The water pH and the optical density were measured with a conductivity meter and a spectrophotometer respectively.

Each isolate and a mixture of the four bacteria were tested in the hospital sewage water, using the same procedure of the PBS assays. For each assay it was done with two replicates.

2.4.3 Assays in PBS with PS and antibiotics

With this assay, it was tested the inactivation of *E. coli* by aPDT in the presence of two antibiotics: ampicillin and chloramphenicol. In the assays with ampicillin it was tested two PS concentrations (2.5 µM and 5 µM) and three concentrations of ampicillin, the MIC concentration (32 $\mu g.mL^{-1}$) (CLSI, 2010) and two lower concentrations (16 $\mu g.mL^{-1}$ and 8 $\mu g.mL^{-1}$).

In the assays with chloramphenicol, it was tested one PS concentration (5 µM) and The MIC concentration (32 $\mu g\ mL^{-1}$) (CLSI, 2010).

For each assay were prepared four goblets: one with bacteria diluted 1:10 in PBS and PS; other with bacteria diluted 1:10 in PBS and PS, plus antibiotic; other with bacteria diluted 1:10 in PBS and PS, plus antibiotic, which was kept in dark conditions, wrapped in aluminium foils (dark control) and other with bacteria diluted 1:10 in PBS , plus antibiotic (light control). The goblets were kept under irradiation 270 minutes and aliquots were taken at 0, 30, 60, 90, 180 and 270 minutes after incubation. The aliquots were diluted in PBS and plated in TSA by incorporation. After 24h at 37ºC, the number of colonies was enumerated and the results were expressed in colony forming units per mL (CFU mL^{-1}). Each assay was done in duplicate, and for each assay two replicates were done.

2.4.4 Assays in PBS with PS and sodium dodecyl sulfate (SDS)

For this test, it was also used the *E. coli* isolate. It was tested one PS concentration (5 μM) and one concentration of SDS (2mM, one quarter of the micellar concentration). The procedure was similar to that of the assays with antibiotics, but instead of antibiotics it was used SDS.

The assays were done in duplicate, and for each assay two replicates were done.

2.4.5 Statistical analysis

The statistical analysis was done with SPSS 17.0. Normal distributions were assessed by Shapiro-Wilk test and the variances homogeneity with the Levene's test. After normality tests were done, ANOVA and the parametric student's t test and the non-parametric Mann-Whitney test were applied to assess the differences between groups.

3. Results

3.1 Resistance profiles of the bacteria strains

The clinical strains employed in this study have diversified resistance profiles (Tab. 1).

A. baumannii is resistant to the gentamicin, tobramycin, meropenem, the combinations amoxicillin/clavulinic acid and piperacillin/tazobactam, ampicillin and penicillin G It is also resistant to cephalothin, cefotaxime and ceftazidime, to ciprofloxacin and levofloxacin and to trimethoprim/sulfamethoxazole. It is sensitive to amikacin and tigecycline.

E. coli is resistant to first, second and third generation cephalosporins cephalothin, cefaclor, cefuroxime–sodium, cefotaxime and ceftazidime. It is also resistant to ciprofloxacin and levofloxacin; to the sulphonamide trimethoprim/sulfamethoxazole, to ampicillin and the penicillin combinations amoxicillin/clavulinic acid and to nitrofurantoin. This strain is sensitive to ertapenem and meropenem and it is a β-lactmase producer.

P. aeruginosa is resistant to ceftazidime, to the penicillin combination piperacillin/tazobactam and to minocycline. This strain is sensitive to imipenem, to amikacin, tobramycin and gentamicin, and has intermediate resistance to the ciprofloxacin.

S. aureus is resistant to penicillin G, to combination piperacillin/tazobactam, levofloxacin, and to erythromycin. This strain is sensitive to gentamicin, tobramycin and vancomycin and to trimethoprim/sulfamethoxazole.

Bacterial strain	*A. baumannii*	*E. coli*	*P. aeruginosa*	*S. aureus*
Ampicillin	Resistant	Resistant	----------	----------
Amikacin	Sensitive	Sensitive	Sensitive	----------
Amoxicillin/Clavulinic Acid	Resistant	Resistant	----------	----------
Cefaclor	----------	Resistant	----------	----------
Cephalothin	Resistant	Resistant	----------	----------
Cefotaxime	Resistant	Resistant	----------	----------
Ceftazidime	Resistant	Resistant	Resistant	----------
Cefuroxime - Sodium	----------	Resistant	----------	----------
Ciprofloxacin	Resistant	Resistant	Intermediate	----------
Erythromycin	----------	----------	----------	Resistant
Ertapenem	----------	Sensitive	----------	----------
Gentamicin	Resistant	Resistant	Sensitive	Sensitive
Imipenem	----------	----------	Sensitive	----------
Levofloxacin	Resistant	Resistant	----------	Resistant
Meropenem	Resistant	Sensitive	----------	----------
Minocycline	----------	----------	Resistant	----------
Nitrofurantoin	----------	Resistant	----------	----------
Penicillin G	----------	----------	----------	Resistant
Piperacillin/Tazobactam	Resistant	Resistant	Resistant	Resistant
Tigecycline	Sensitive	----------	----------	----------
Tobramycin	Resistant	Resistant	Sensitive	Sensitive
Trimethoprim/ Sulfamethoxazole	Resistant	Resistant	----------	Sensitive
Vancomycin	----------	----------	----------	Sensitive
Broad spectrum β-lactmase	----------	β-lactmase Producer	----------	----------

Tab. 1: Drug resistance profile for the studied strains (--- not done).

3.2 Photoinactivation of bacteria in PBS

The aPDT was effective against all bacteria, Gram-positive and Gram-negative, leading to a reduction in colony forming units (CFU) between 6 to 8 log, after 270 minutes of irradiation.

For *E. coli* (Fig. 3 A), there was a gradual inactivation to the limit of detection after 180 minutes. For *P. aeruginosa* (Fig. 3 B) a 7 log inactivation was observed after 270 minutes and the bacterial reduction started only after 30 minutes of irradiation. For *A. baumannii* (Fig. 3 C) it was not observed the inactivation to the limit of detection, 2 log of bacteria were not inactivated after 270 minutes of irradiation. The greater decrease in bacterial number for these bacteria was observed only after 90 minutes of irradiation. The reduction of bacterial density for *S. aureus* (Fig. 3 D) was observed sooner, after 30 minutes of irradiation it was observed already a decrease of 3 log and after 90 minutes of irradiation a decrease of 6 log. For the mixture of bacteria the greater reduction was observed between 30 and 180 minutes of irradiation. It was not observed a significant reduction of the bacteria between 180 and 270 minutes of irradiation.

Dark and light controls did not exhibit reduction in bacterial number after the incubation period.

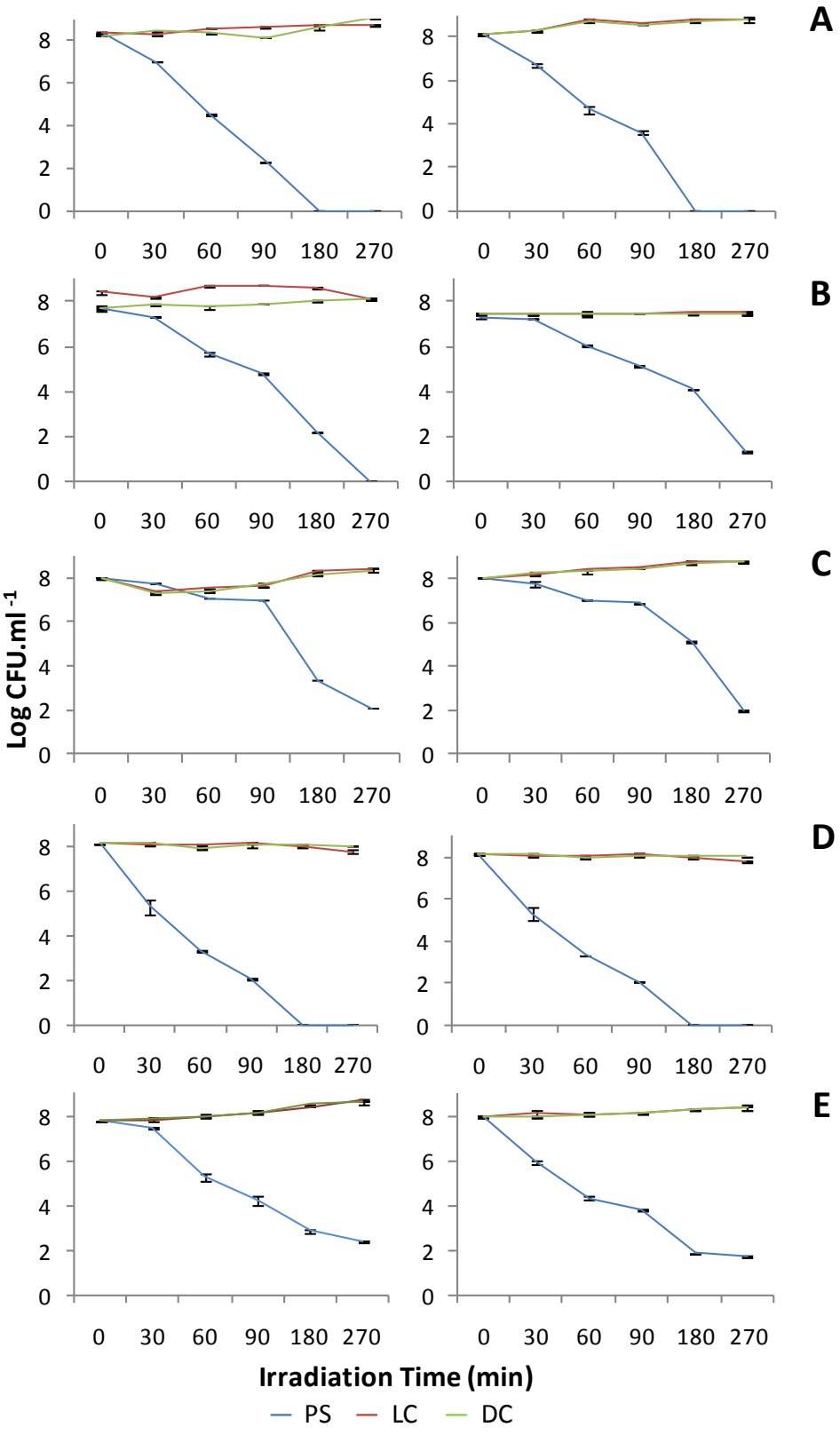

Fig. 3: Photoinactivation of *E. coli* (A), *P. aeruginosa* (B), *A. baumannii* (C), *S. aureus* (D) and a mixture of all four bacteria (E), in PBS, after 30, 60, 90, 180 and 270 minutes of irradiation (PS, Photosensitizer; LC, Light Control; DC, Dark Control). It was done 2 independent assays, where, each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations

3.3 Bacterial photoinactivation in sewage water

The pH of the two first samples of sewage was similar, but the third samples had a higher value of pH. The value of optical density after filtration by 0.2 µM was higher for the second date (Tab. 2). The second water sample was more yellow than the others.

Collection date	pH	Optical density
10 Nov	7.272	0.004
24 Nov	7.145	0.008
30 Nov	8.268	0.004

Tab. 2: pH and optical density values for the three samples of hospital sewage water

The patterns of bacterial photoinactivation were different for the three dates, namely between 180 to 270 minutes ($p<0.05$) (Fig. 4). The difference between the three samples was not significant for the other times of irradiation.

For all the bacteria the major reduction was observed after 30 minutes of irradiation, with reduction of at least 4 logs. The reduction to the limits of detection rarely was observed even for *S. aureus. P. aeruginosa* reach the reduction to the limit of detection in two of the three dates. For the mixture of bacteria the pattern of inactivation was similar to those observed for the isolated bacteria, the major reduction of bacterial number was observe after 30 minutes of irradiation and the reduction to the detection limit was not observed in none of the three dates, remaining approximately 1,5 log of bacteria after irradiation.

Dark and light controls do not exhibit any reduction in bacterial counts during the irradiation period.

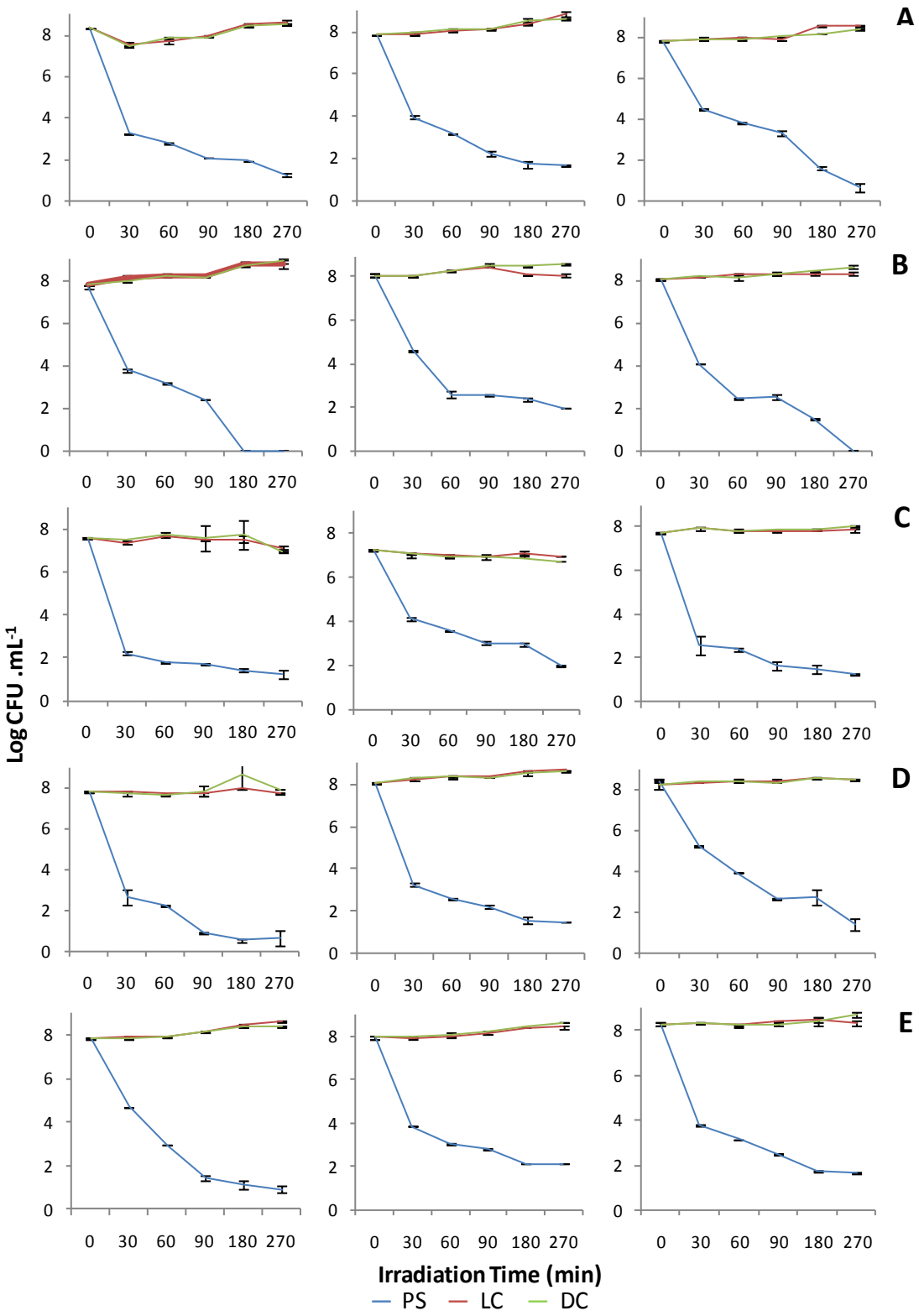

Fig. 4: Photoinactivation of E. coli (A), P. aeruginosa (B), A. baumannii (C), S. aureus (D) and a mixture of bacteria (E), in sewage water, after 30, 60, 90, 180 and 270 minutes of irradiation (PS, Photosensitizer; LC, Light Control; DC Dark Control). It was done 3 independent assays, where each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.

3.4 Comparison between photoinactivation of bacteria in PBS and in sewage water

In general, the pattern of photoinactivation in PBS and in sewage water was significantly different for all the bacteria tested and for the mixtures of bacteria. For *E. coli*, the difference between the results obtained in PBS and in hospital sewage waters was significant after 30 minutes of incubation to 270 minutes (ANOVA, p <0.05)., while for *P. aeruginosa*, the difference between the results obtained in PBS and in hospital sewage waters was significant only between 30 and 180 minutes (ANOVA, p <0.05). The photoinactivation of *A. baumannii* was also significantly different right after 30 minutes to 270 minutes of irradiation (p <0.05). For *S. aureus*, the difference between the two types of assays was significant at 30 minutes of irradiation and after 180 and 270 minutes of irradiation (p <0.05). For the mixture of the bacteria, there is a significant difference between the results obtained in PBS and in hospital sewage in the sampling times of 30 and 90 minutes (p<0.05). After 60 minutes of irradiation there is no statistical difference between PBS and hospital residual waters (p>0.05)

3.5 Photoinactivation of bacteria by aPDT and antibiotics

The photoinactivation with 5 µM of PS in the presence of ampicillin at MIC concentration (32 $\mu g.mL^{-1}$) was higher than when the antibiotic was not added (Fig. 5 A). The major reduction on *E. coli* was achieved after 60 to 90 minutes of irradiation. It was observed a difference of about 1.5 log between the inactivation of *E. coli* by aPDT alone and aPDT together with ampicillin, after 180 minutes of irradiation and after 270 minutes this different was approximately 2 log. The difference between the results obtained with aPDT alone and aPDT combined with 32 $\mu g.mL^{-1}$ was statistically significant for the sampling times of 180 minutes and 270 minutes (p <0.05). At the end of 180 minutes the bacterial reduction was approximately 6 log for aPDT alone and 7 log for aPDT combined with ampicillin and at the end of 270 minutes the bacterial reduction remain in the 6 log for aPDT alone and for aPDT combined with ampicillin increased for 8 log reduction.

When the concentration of ampicillin was decreased to 16 $\mu g.mL^{-1}$ (Fig. 5 B), the major reduction was observed after 180 minutes of irradiation, being the

difference between aPDT and aPDT combined with ampicillin significantly different after 180 minutes and 270 minutes of incubation ($p<0.05$). At the end of 180 minutes the bacterial reduction was approximately 4 log for aPDT alone and 6 log for aPDT combined with ampicillin and at the end of 270 minutes the bacterial reduction was 5.5 log for aPDT alone and 6.5 log for aPDT combined with ampicillin.

At 8 $\mu g.mL^{-1}$ ampicillin (Fig.5 C), the pattern of inactivation was similar to that obtained without antibiotic (p >0.05). At the end of the experiment the difference in bacterial inactivation between the two assays was of 0.5 log, with the higher reduction value for aPDT in the presence of the antibiotic.

In the light and dark control samples, it was not observed inactivation of *E. coli* during the 270 minutes of incubation

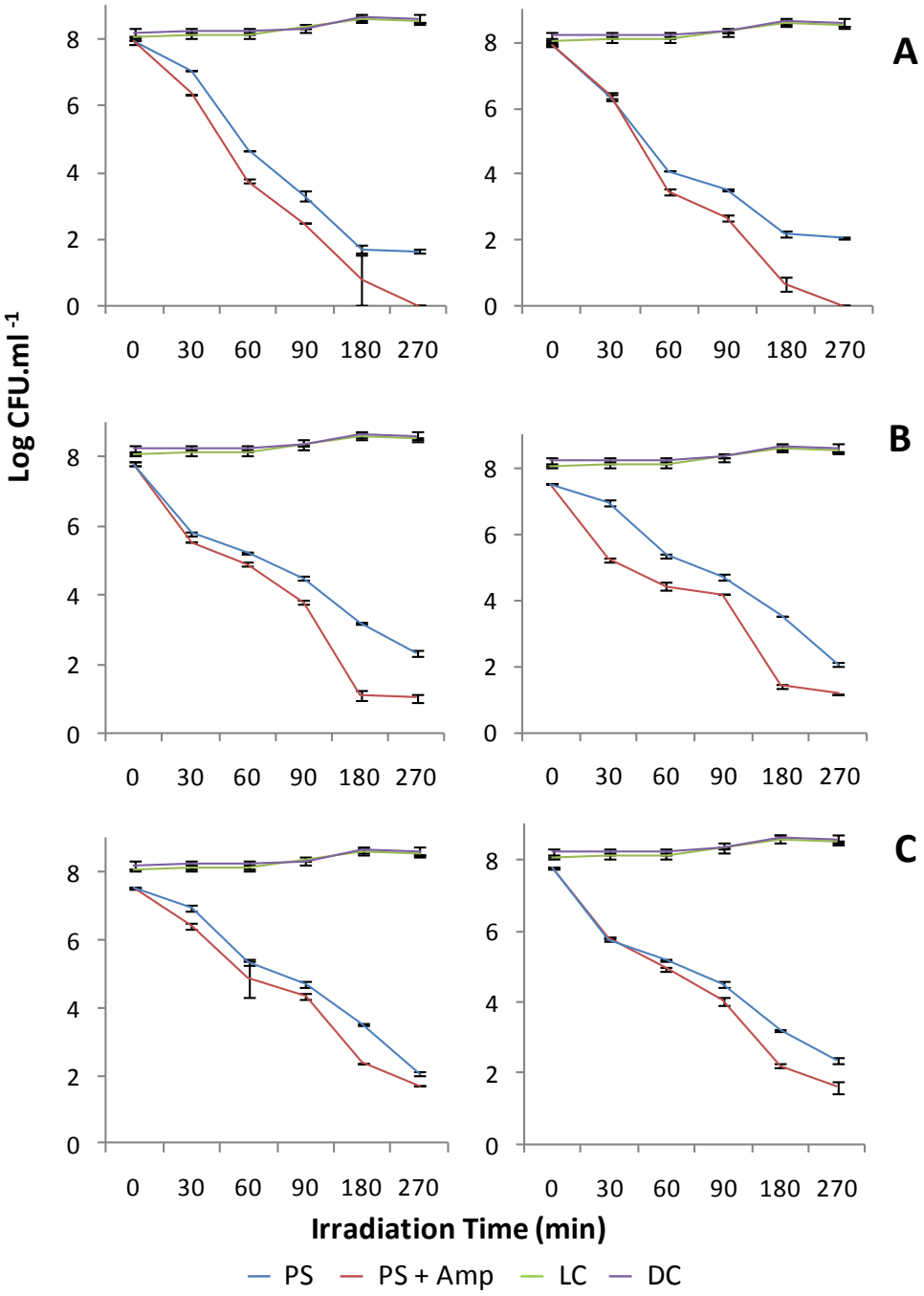

Fig. 5: Photoinactivation of *E. coli* in PBS, after 30, 60, 90, 180 and 270 minutes of irradiation, with ampicillin concentrations of 32 µg.mL^{-1} (A), 16 µg.mL^{-1} (B) and 8 µg.mL^{-1} (C) (PS, Photosensitizer; PS + Amp, Photosensitizer with ampicillin; LC, Light Control; DC, Dark Control). Two independent assays were done, in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations

When the PS concentration was reduced to 2.5 µM in the presence of 32 $\mu g.mL^{-1}$ of ampicillin (Fig. 6), at the end of 270 minutes, the inactivation pattern was similar to that obtained when no antibiotic was added (p >0.05), being the difference between the two less than 1 log.

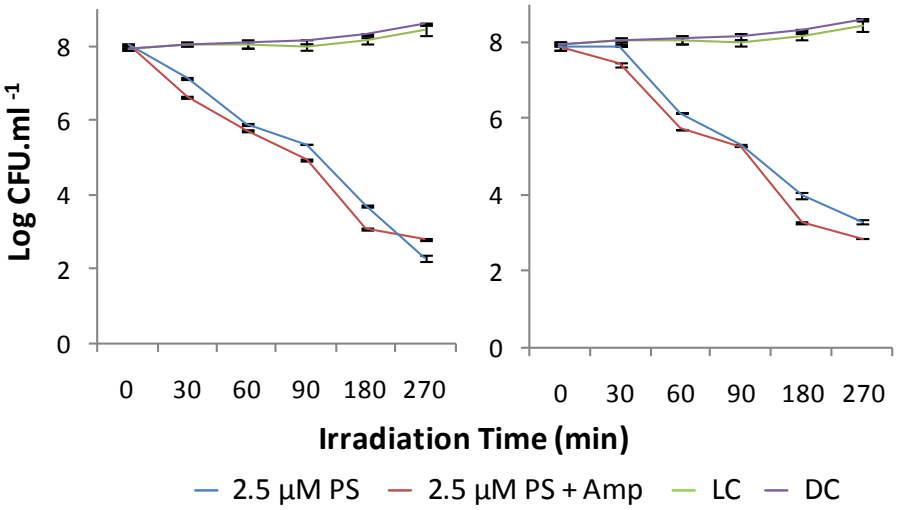

Fig. 6: Logarithmic inactivation of *E. coli* in PBS, after 30, 60, 90, 180 and 270 minutes of irradiation, with a concentration of PS of 2.5 µM and an ampicillin concentration of 32 $\mu g.mL^{-1}$ (PS, Photosensitizer; PS + Amp, Photosensitizer with ampicillin; LC, Light Control; DC, Dark Control). It was done two independent assays in which each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations

When aPDT with 5 µM PS was used in combination with 32 µg mL^{-1} of chloramphenicol (Fig. 7), the bacterial reduction was higher than that obtained only in the presence of PS, being the difference significantly different after 270 minutes of irradiation (p <0.05). The difference between aPDT alone and aPDT combined with chloramphenicol after 270 minutes of irradiation was in average 2 logs.

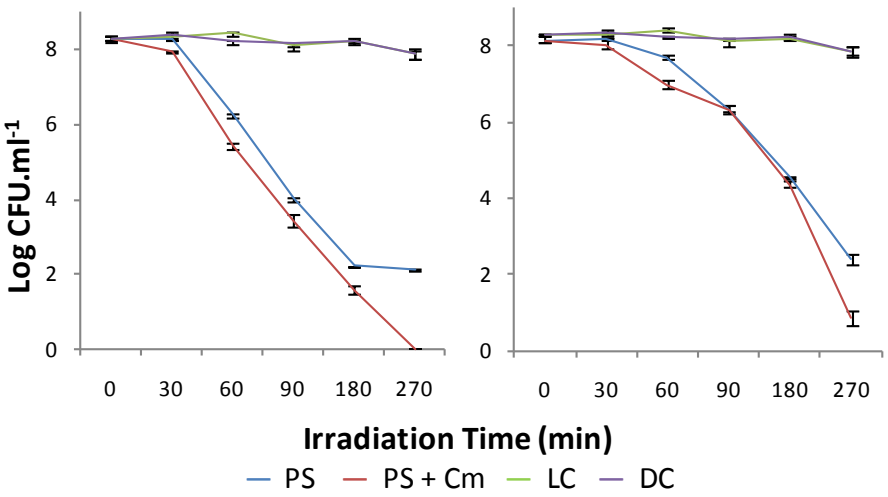

Fig. 7: Photoinactivation of *E. coli* in PBS, after 30, 60, 90, 180 and 270 minutes of irradiation, with a PS concentration of 5µM and a chloramphenicol concentration of 32 µg.mL-1 (PS, Photosensitizer; PS + Cm, Photosensitizer with chloramphenicol; LC, Light Control; DC, Dark Control). Each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.

3.6 Photoinactivation of bacteria by aPDT and SDS

The inactivation of *E. coli* by aPDT was not significantly different (p >0.05) from that observed in the presence of 2 mM of SDS (Fig. 8) during the whole assay.

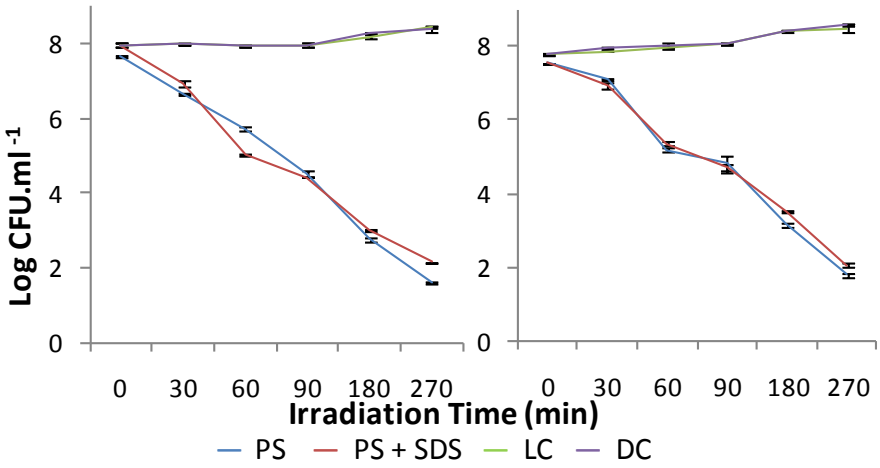

Fig. 8: Photoinactivation of *E. coli* in PBS, after 30, 60, 90, 180 and 270 minutes of irradiation, with 2mM of SDS (PS, Photosensitizer; PS + Cm, Photosensitizer with chloramphenicol; LC, Light Control; DC, Dark Control). Each value corresponds to the mean ± standard deviation of two replicates. Error bars correspond to standard deviations.

4. Discussion

Antibiotic resistance is a complex, continually evolving problem which is often difficult to put into perspective (Barker, 1999). In recent years, facing the growing costs of research on new antimicrobials, and despite the increasing frequency and severity of antimicrobial resistance, the pharmaceutical industry has diminished its investment in this field, turning to more profitable drugs. This has dramatically weakened the so called pipeline for new antibiotic compounds, particularly against Gram-negative bacteria. It is therefore, a major goal of the present research to find new approaches to combat microbial diseases, as well as to increase the efficacy of classical antimicrobial compounds, without administering them in large dosages.

The results of this study showed that (1) aPDT can efficiently inactivate multidrug resistant bacteria; (2) bacterial photoinactivation in hospital sewage water is faster than in PBS in the first 30 minutes of treatment, but after 270 minutes of treatment the efficiency were not significantly different; (3) the combination of aPDT with antibiotics increase the efficiency of bacterial inactivation; (4) the combination of aPDT with SDS did not lead to a higher photoinactivation of bacteria.

The studied cationic porphyrin Tetra-Py^{+}-Me at the concentration of 5 μM, when irradiated with white light (40 W m^{-2}), efficiently inactivated both gram-positive and gram-negative bacteria (between 6 and 8 log) in PBS, and was also efficient when the bacteria were tested all together (approximately 6 log of inactivation).

In general, multidrug resistant bacteria were inactivated to the detection limits (≈ 8 log) but the profile of the photoinactivation process varied among the bacterial strain (ANOVA, $p<0.05$). As previously reported (Hamblin et al., 2004; Jori et al., 2004; Arrojado et al., 2011) the Gram (+) bacterium was inactivated faster than Gram (-) bacteria. The major reduction on cell viability occurred after 30 min of irradiation, causing approximately 3 log decrease for the Gram (+) bacteria, but, in general, the major reduction for Gram (-) bacteria occurred only after 60-180 min of irradiation, causing approximately 3-4 logs decrease. All the multidrug resistant bacteria, with the exception of *A. baumannii*, were completely inactivated after 270 min of exposure to the white light in PBS. For *A. baumannii* after 270 min of exposure

around 2 logs of cell survived to the photoinactivation process. The difference in the susceptibility of Gram (+) and Gram (-) bacteria to the photoinactivation process is easily explained by the different structure of the cell wall. In gram-positive bacteria, the wall is easily crossed by all types of photosensitizers (Hamblin et al., 2004; Jori et al., 2004; Arrojado et al., 2011). The different profiles of inactivation among the Gram (-) bacteria can be also related to differences in the cell wall. Although all Gram (-) bacteria have a thin layer of peptidoglycan and an asymmetric outer membrane of lipidic composition, the composition of the lipopolysaccharide of the external outer membrane varies among bacteria (Dahl et al., 1987; Jori et al., 2004; Arrojado et al., 2011). The lower inactivation of *A. baumannii* can be related to the fact that this bacterium has fewer and smaller porins than other Gram-negative bacteria, thereby decreasing cell permeability and increasing antibiotic resistance (Villa et al., 2007) and also probably difficult the porphyrin penetration. In fact, the strain used in this study is more resistant to antibiotics than the other isolates tested in this study. *A. baumannii* was resistant to 86 % of the tested antibiotics*, E. coli* to 78 %, *P. aeruginosa* to 40 % and S. aureus to 50 %. Differences in the effectiveness of aPDT among bacteria were also showed in other studies (Grinholc et al., 2007; Arrojado et al., 2011).

The patterns of bacterial photoinactivation in hospital wastewater samples were different from those obtained in PBS for all bacteria ($p<0.05$). Most of the bacterial inactivation occurred during the first 30 minutes, resulting in, at least, 3 logs reduction after this time. However, after 270 minutes of irradiation the difference between the two types of samples was not significant and, in general, the efficiency of the photoinactivation process was even slowly lower in the sewage water ($p<0.05$). Some studies have shown, however, that microbial inactivation by aPDT in environmental waters is not as effective as in laboratory conditions in which microorganisms are suspended in buffer solutions. Alves et al (2011) verified that the inactivation of *Vibrio fischery* by 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin triiodide in laboratory conditions, using PBS, was more effective than in water from aquaculture tanks. The same authors showed that the removal of particulate suspended matter from the aquaculture water increases the success of aPDT. Arrojado et al. (2011) find also that it is more difficult to inactivate

the natural bacterial communities in aquaculture waters than pure cultures of bacteria isolated from aquaculture systems in PBS. The authors suggested that the difficulty in the inactivation of bacteria in aquaculture waters could be due to differences in bacterial community structure but can be also attributed to differences in suspended matter quantity and quality. Jemli and Alouini (2002) concluded also that the suspended solids (turbidity) were the parameter that most influenced on the efficiency of the photochemical process of helminth eggs in wastewater by *meso*-tetrakis(1-methylpyridinium-4-yl)porphyrin tetratosylate. To the best of my knowledge this is the first study that the efficiency of photoinactivation was higher in environmental waters than in buffered solutions as PBS. However, as the sewage waters were filtered by 0.2 µM membranes before the photoinactivation assays, most of the suspended matter was removed.

The dissimilarity could be due the existence of diverse kind of dissolved compounds in the hospitals wastewaters, like pharmaceuticals (Andreozzi, et al., 2003), heavy metals, polycyclic aromatic hydrocarbons, chlorinated dioxins, furans, pesticides and detergents (Elmolla et al., 2008), which, some of them, can stimulate the photoinactivation process. As in the light and dark controls of the photoinactivation assays in sewage waters were not observe any reduction in the bacterial number for the four bacteria, these dissolved compounds do not affected directly the bacteria.

As the four bacteria tested in this work are all multi-resistant to antibiotics it was tested the aPDT in the presence of antibiotics in order to understand if a synergistic effect of aPDT and antibiotics could explain the stimulation of aPDT in sewage waters containing several families of antibiotics. Being the main target of aPDT the external structures of the bacteria, cell membrane and cell wall, are destabilized allowing an easier enter of the antibiotics into the resistant cells (inhibiting for instance protein synthesis that can be the antibiotic target, like for chloramphenicol) and, naturally, improving the action of antibiotics which have the external structures as its target (like for ampicillin). By this way, the antibiotic effect can be also stimulated. This synergistic effect between aPDT and antibiotics was already detected by Malik and Nitzan (1995) for the inactivation of both Gram-positive multi-resistant bacteria (*S.*

aureus) and Gram-negative multi-resistant bacteria (*E. coli*) and for the disruption of *S. aureus* biofilms (Di Poto et al., 2009).

On the other hand, the porphyrin and the antibiotic can be combined to form a new molecule that acts as a truly new antibacterial. This hypothesis is supported by Xing et al. (2011) who developed a vancomycin-porphyrin conjugate with enhanced properties in the photoinactivation of vancomycin-sensitive and vancomycin-resistant enterococci.

The results of this study showed that the photodynamic activity combined with antibiotics, lead to a higher inactivation of *E. coli* than aPDT alone. With 32 μg mL^{-1} and 16 μg mL^{-1} of ampicillin it was obtained a greater inactivation with the combination of aPDT and antibiotics. This effect was not observed when 8 μg mL^{-1} of antibiotic was used. For the tested concentration of chloramphenicol (32 μg mL^{-1}) the efficiency of the bacterial inactivation was also increased after the photoinactivation treatment. However, at this moment it is not yet possible to discriminate between the two hypotheses, synergistic effect or the formation of a new molecule. Nevertheless, the results of this study suggest that porphyrins can be exploited to increase the efficacy of antibiotics, and to reduce the dosages of antibiotic concentration needed to combat infections.

As sewage waters have high concentrations of detergents and these chemicals are used in laboratory to lise bacterial cells due to their high content in lipids in membranes it was also tested the aPDT in the presence of SDS in order to explain the different efficiency of aPDT in sewage waters and in PBS. The difference between aPDT alone and in combination with SDS was however not significant (p >0.05). This means that the effect of the SDS on the bacterial external structures is not enough, but the effect of 4 mM of SDS was similar (data not shown), or that the effect of aPDT on the external structures is enough to destabilize the bacterial cells and the effect of the SDS is, by this way, masked. Other possible explanation is the potential interaction of the SDS with the porphyrin. Some dyes may form dye-rich premicellar aggregates in dilute surfactant solutions, detected by modified absorption spectra for aggregated dyes when compared with monomeric dyes. Surfactant-dye interaction is common for oppositely charged dye-detergent pairs (Barber et al., 1991)

From the data obtained in this study, it can be concluded that aPDT has the potential to be an effective alternative for the treatment of multidrug-resistant infections and can be combined with antibiotics to enhance its effectiveness.

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