Universidade de Aveiro – Departamento de Biologia
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Cristiana Paula Martins da Silva Oliveira

Photodynamic Therapy in the Inactivation of Bacteriophages with Porphyrin and Potentiators in Wastewater

Terapia Fotodinâmica na Inativação de Bacteriófagos com Porfirina e Potenciadores em Águas Residuais

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Professora Doutora Adelaide Almeida, Professora com Agregação do Departamento de Biologia da Universidade de Aveiro e sob a coorientação da Doutora Carla Pereira, Investigadora do Departamento de Biologia da Universidade de Aveiro

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PALAVRAS-CHAVE

Terapia fotodinâmica antimicrobiana, formulação porfirínica, águas residuais, bacteriófago tipo T4, iodeto de potássio, peróxido de hidrogénio

RESUMO

Os vírus patogénicos são frequentemente introduzidos nas águas marinhas e estuarinas através da descarga de esgoto tratado e não tratado, uma vez que os tratamentos atuais não inativam os vírus presentes nas águas residuais (WW), afetando a qualidade das águas recetoras e, consequentemente, a saúde humana. Nos tratamentos convencionais, a remoção de constituintes nocivos consiste no uso de métodos químicos, físicos e biológicos. Geralmente, a WW de áreas urbanas é tratada secundariamente e não terciariamente. Embora o efluente secundário contenha altas concentrações de microrganismos, o efeito da diluição na água torna-o aceitável em termos de indicadores de qualidade. A cloração é o método mais comum usado para garantir a segurança microbiológica em efluentes tratados terciariamente. No entanto, a sua utilização maciça, tanto na forma de cloro livre como combinada, pode levar à formação de subprodutos químicos como resultado da reação com a matéria orgânica presente nos efluentes, sendo esses produtos químicos tóxicos para os organismos aquáticos, apresentando riscos para a saúde. Os métodos convencionais são limitados e podem não ser adequados para manter os níveis de qualidade especificados nas diretrizes. As porfirinas quando usadas como fotossensibilizadores (PS) na terapia fotodinâmica (PDT) podem ser desinfetantes promissores para a inativação de microrganismos patógenicos, pois são eficazes na inativação de microrganismos sem formação de produtos tóxicos. Alguns estudos mostraram efeito potenciador de alguns PS usados em terapia fotodinâmica antimicrobiana (aPDT) quando estes são usados em combinação com iodeto de potássio (KI) e peróxido de hidrogénio (H_2O_2). O principal objetivo deste estudo foi avaliar a eficácia da aPDT de um PS baseado numa formulação de baixo custo constituída por cinco porfirinas catiónicas (Form) e o seu efeito potenciador por KI e H_2O_2 na inativação de um bacteriófago tipo T4. As experiências foram realizadas em solução salina tamponada com fosfato e em água residual contaminada filtrada e não filtrada. Os ensaios de aPDT em WW filtrada (tamanho do poro de 0,45 µm) foram realizados com diferentes concentrações de Form (1,0 a 10 µM). Numa segunda fase foi avaliado o efeito do KI (100 mM) na ação fotodinâmica da FORM (1,0 a 10 µM). Os resultados dessas experiências demonstraram que a Form é eficiente no tratamento de WW filtrada e que a eficácia da fotoinativação de bacteriófagos está correlacionada com a concentração do PS usado. Quando combinada com o KI, a Form é claramente menos eficaz na inativação do bacteriófago. Para avaliar se a matéria orgânica presente na água influencia a eficiência do PS, a WW foi filtrada usando três membranas com tamanho de poros diferentes (0,45, 0,30 e 0,22 µm). Os resultados mostraram que o aumento da matéria orgânica promove uma diminuição significativa na eficiência da Form. Para avaliar se a eficiência da aPDT para inativar bacteriófagos é mantida quando os tratamentos são realizados em WW não filtrada, o efeito da Form sozinha (10 µM) e combinado com H_2O_2 (2, 5 e 9%) em WW não filtrada foi avaliado. A Form por si só provou ser um PS eficiente para fotoinativar o bacteriófago em

RESUMO

(Continuação)

WW não filtrada, mas a presença de H_2O_2 aumentou significativamente o efeito fotodinâmico. A Form pode ser uma alternativa eficaz para controlar vírus na WW, principalmente se combinada com H_2O_2.

KEYWORDS

Antimicrobial photodynamic therapy, porphyrinic formulation, wastewater, bacteriophage T4-like, potassium iodide, hydrogen peroxide

ABSTRACT

Pathogenic viruses are frequently introduced into marine and estuarine waters through the discharge of treated and untreated sewage, since current treatments are unable to provide virus-free wastewater (WW) effluents, affecting the receiving waters quality and, consequently, human health. The removal of harmful constituents by the conventional treatments comprises a combination of chemical, physical and biological methods. Usually, WW from urban areas is secondarily, rarely tertiary, treated. Although the secondary effluent contains high concentrations of microorganisms, the effect of water dilution makes it acceptable in terms of quality indicators. In tertiary treatment, chlorination is the most common method used to ensure microbiological safety in tertiarily treated effluents. However, its massive utilization, both in free and combined chlorine forms, may lead to the formation of chemical disinfection by-products though the reaction with organic matter present in the effluents, being those chemicals toxic to aquatic organisms, representing potential health hazards. Unfortunately, these conventional methods are limited and may not be adequate to reach the quality levels specified by the guidelines. Photodynamic therapy (PDT) with porphyrins may be a promising approach for the inactivation of pathogens as they are effective in inactivating microorganisms without the formation of potentially toxic products. Some studies have reported an enhancer effect on antimicrobial photodynamic therapy (aPDT) by the combined used of some photosensitizer (PS) with potassium iodide (KI) and hydrogen peroxide (H_2O_2). The main objective of this study was to evaluate the aPDT efficacy of a PS based on a low-cost formulation constituted by five cationic porphyrins (Form) and its potentiation effect by KI and H_2O_2 in the inactivation of *a* T4-like bacteriophage in WW. The experiments were done in phosphate buffered saline and in filtered and non-filtered contaminated wastewater. The aPDT assays in filtered WW (0.45 µm pore-size) were performed with different concentrations of Form (1.0 to 10 µM). In a second phase was evaluated the effect of KI (100 mM) in the photodynamic action of Form (1.0 to 10 µM). The results of these experiments demonstrated that Form is efficient in filtered WW treatment and that the efficacy of bacteriophage photoinactivation is correlated with the concentration of the used PS. When combined with KI, the Form is clearly less effective to inactivate the bacteriophage. To evaluate if the organic matter present in water influences the efficiency of PS, the WW was filtered using three different pore-sized membranes (0.45, 0.30 and 0.22 µm). The results demonstrated that the increase of organic matter promote a significant decrease in the efficiency of Form. In order to evaluate if the efficiency of aPDT to inactivate bacteriophages is maintained when the treatments are performed in non-filtrated WW, the effect of Form alone (10 µM) and combined with H_2O_2 (2, 5 and 9%) in non-filtered WW was evaluated. The Form alone proved to be an efficient PS to photoinactivate the bacteriophage in non-filtered WW, but the presence of H_2O_2 enhanced the photodynamic effect. The FORM can be an effective alternative to control viruses in WW, particularly if combined with H_2O_2.

Contents

LIST OF ABBREVIATIONS AND ACRONYMS

AOP	Advanced Oxidation Process
aPDT	Antimicrobial Photodynamic Therapy
CFU	Colony Forming Units
Di-Py(+)-Me adj	5,15-bis(1-methylpyridinium-4-yl)-10,20-bis(pentafluorophenyl)porphyrin di-iodide
Di-Py(+)-Me opp	5,10-bis(1-methylpyridinium-4-yl)-15,20-bis(pentafluorophenyl)-porphyrin di-iodide
DC	Dark Control
DLA	Double-Layer Agar
LC	Light Control
MB	Methylene Blue
MDR	Multidrug Resistant
MO	Microorganism
Mono-Py(+)-Me	5-(1-methylpyridinium-4-yl)-10,15,20-tris(pentafluorophenyl)-porphyrin iodide
NSAID	Nonsteroidal Anti-inflammatory Drug
PBS	Phosphate Buffered Saline solution
PDI	Photodynamic Inactivation
PDT	Photodynamic Therapy
PFU	Plaques Forming Units
PPCP	Pharmaceutical and Personal Care Product
PS	Photosensitizers
SMP	Soluble Microbial Products
Tetra-Py(+)-Me	5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide
Tri-Py(+)-Me	5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
ROS	Reactive Oxygen Species
WWTP	Wastewater Treatment Plant

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OBJECTIVES

The main objective of this study was to evaluate the antimicrobial photodynamic therapy (aPDT) efficacy of a photosensitizer (PS) based on a low-cost formulation constituted by five cationic porphyrins (Form) and its potentiation effect by KI and H_2O_2 in the inactivation of bacteriophages in wastewater. For this, a well characterized bacteriophage, bacteriophageT4-like, was used. The experiments were done in phosphate buffered saline, filtered wastewater and non-filtered wastewater contaminated with bacteriophage T4-like.

The specific objectives can be summarized as:

- To evaluate the potential effect of Form and potentiation effect by KI in the inactivation of bacteriophage T4-like in filtered wastewater;
- To evaluate the potential of Form and potentiation effect by H_2O_2 in the inactivation of bacteriophage T4-like in non-filtered wastewater;
- To evaluate if the organic matter and native microorganisms presents in wastewater can be influence the aPDT efficiency;
- Development of a protocol for control of viral contamination in wastewater, using porphyrinic compounds.

CHAPTER 1 - GENERAL INTRODUCTION

1.1) IMPACT OF WASTEWATER EFFLUENT

Safe water and proper sanitation are indispensable factors to sustain life. Nevertheless, currently there are 884 million and an additional 2.5 billion people lacking improved water sources and sanitation, respectively (WHO, 2012). This crisis is further compounded by factors such as increasing poverty, accelerated population growth and rapid urbanization coupled with hydrological variability and climate change (WHO, 2012). These socio-economic and environmental factors place even further stress on the deteriorating water and sanitation infrastructure, more so in developing regions, where billions are still at risk of water, sanitation and hygiene related diseases (WHO, 2012).

Reliable wastewater treatment systems serve as a good indication of the level of development within a municipality as well as community health, with the degree and quality of wastewater determining the impact of these treatment plants on surrounding water sources into which it is released (Department of Water Affairs, 2011). Each country undertakes to protect its water resources with water policies, monitoring, and treatment strategy (Department of Water Affairs, 2011). Although water is normally a recyclable resource, it requires careful management and protection because it is vulnerable to overexploitation and pollution (Osuolale *et al.* 2017). Avoiding the contamination of water assets and ensuring human well-being by protecting water supplies against the spread of pathogenic organisms are the two principal purposes behind the treatment of wastewater (Osuolale *et al.* 2017). Over the last few years, the quantity of municipal wastewater produced has drastically increased due to the constant increase in population numbers together with an increased dependence on diminishing water resources (UNICEF and WHO, 2012). This coupled with the discharge of inefficiently treated wastewater into surrounding surface water sources serve as a direct threat, not only to the macro- and microflora and fauna present, but also the human health (UNICEF and WHO, 2012). Thus, the constant monitoring of the operational status of existing wastewater treatment plants as well as increasing emphasis on environmental and water resource health has become key factors in determining the quantity and quality of wastewater generated by respective municipalities (UNICEF and WHO, 2012).

Treated sewage is a major source of human-derived microorganisms in the urban water environment, including potential pathogens that may survive the treatment process (Newton *et al.* 2019). The microorganisms from water contact are recognized as a significant component of an individual's cumulative interaction with the environment, which is thought to be responsible for 70%–90% of all human illnesses (Newton *et al.* 2019). More specifically, untreated sewage escaping sewer systems ends up in groundwater or surface waters within city limits, where people can be exposed through recreation or intrusion into drinking water distribution systems (Vikesland *et al.* 2017). It is estimated there are 90 million cases of waterborne illness in the U.S. per year from recreational water exposures, costing $2.2– 3.7 billion (Vikesland *et al.* 2017). The persistence and/or decay of common human pathogenic organisms in surface waters have been evaluated, however, the influence of resident microbiota on pathogen decay is rarely considered, and the prevalence of emerging wastewater infrastructure-associated pathogens such as *Arcobacter* spp. remains relatively understudied (Newton

et al. 2019). The health risk posed by improper sanitation is not restricted to waterborne pathogens; treated and untreated sewage carries with it a plethora of chemicals, hormones, and antibiotic resistant bacteria and resistance conferring genes (Newton *et al.* 2019). Given the incredibly high flux of urban microorganisms into natural water bodies, widespread colonization of natural systems by wastewater microorganisms or dissemination of their associated genes seems likely (Vikesland *et al.* 2017). For example, a continental-scale assessment of Chinese estuaries revealed 18 ARGs (antibiotic resistance genes) were present in all sediment samples over a 4000 km coastline, and the authors associated these genes directly to human activities (Newton *et al.* 2019). In other study, the exact gene variants from common wastewater infrastructure organisms (*e.g. Acinetobacter*, *Legionella*, *Neisseria*) were identified in both wastewater treatment plant (WWTP) effluent and receiving water sediments, and these genes increased as one neared the discharge points (Vikesland *et al.* 2017).

1.2) SOURCES OF WASTEWATER

Wastewater is defined as any storm water runoff, as well as industrial, domestic or hospital sewage or any combination thereof carried by water (CIDWT, 2009). The type and volume of wastewater generated is determined by both, population numbers and the combination of surrounding domestic, recreational and industrial activities, all of which affect discharge patterns as well as the chemical status of the treated effluent (CIDWT, 2009). In order to set up an efficient waste management system, proper identification and characterization of the influent entering a wastewater treatment plant is essential (Mara, 2004). This is based on the physical, chemical and biological characteristics of the influent; the immediate and downstream effect on the surrounding environment into which the wastewater will be discharged as well as the currently laid out environmental and discharge standards (Mara, 2004).

The main types of wastewater are domestic, industrial, agricultural, urban and hospital sewage. The composition of the wastewater varies significantly depending on its source (WWAP, 2017; Bartolomeu *et al.* 2018). Though, the main wastewater pollutants can exist in all wastewater sources, for example: suspended solids, biodegradable organic matter like phosphorus compounds, non-biodegradable matter such as pesticides and detergents, metals, inorganic dissolved solids and microorganisms, including pathogens (WWAP, 2017; Bartolomeu *et al.* 2018). Reliant on these variations of concentrations, some wastewaters may be less hazardous than others, yet, even at lower levels, these pollutants may cause long-term issues, such as: infections transmitted to humans or animals by pathogens; cancer or embryo/fetal effects by toxic organic compounds; and even the increase of salinity and sodium content in soil, leading to the decrease of soil permeability (WWAP, 2017; Bartolomeu *et al.* 2018). Domestic sewage is a complex mixture containing water together with organic and inorganic constituents and large numbers of pathogenic bacteria as well as viruses and parasites (US EPA, 2003). Hospital sewage is that coming from the hospitals and medical centres and includes sewage and wastewater resulting from the cleaning of laboratories and other facilities (US EPA,

2003). Antibiotics, disinfectants and antibiotic-resistant bacteria are the major constituents in these wastes (due to their major use in hospital practice) (Pauwels and Verstraete, 2006; Jury *et al.* 2010). The agricultural runoff is now becoming increasingly important due to the high quantities of pesticides and fertilizers being used, ultimately contributing to surface water eutrophication (Department of Water Affairs, 2011). The composition of industrial wastewater varies based on the type of surrounding industry together with respective contaminant and pollutant composition with general classification into inorganic and organic industrial wastewater (Rosenwinkel *et al.* 2011).

1.3) MICROBIAL PATHOGENS IN WASTEWATER

Microbial pathogens are among the major health problems associated with wastewater (Naidoo *et al.* 2014). The high concentration of microorganisms (Table 1) may create a severe health risk when raw wastewater is discharged into receiving waters (Henze, 2008). The microorganisms in wastewater come mainly from human's excreta, as well as from livestock and agriculture (Henze, 2008). While some of these microorganisms play an important role in the decomposition of waste and are considered an integral component of organic matter, others are pathogenic, or disease-carrying, and pose a threat to public health (Naidoo *et al.* 2014). Microbial pathogens which can be potentially present in wastewater can be divided into three groups, namely, viruses, bacteria and pathogenic protozoan/helminths (Table 1) (Naidoo *et al.* 2014). The majority of these microorganisms are enteric in origin, that is, they are excreted in faecal matter, contaminate the environment and then, the new hosts through ingestion (Naidoo *et al.* 2014).

Table 1. Microbial indicators of wastewater quality and pathogenic organisms associated with waterborne diseases and common sources of contamination (Naidoo *et al.* 2014).

	Microorganisms	**Diseases**	**Source**	**Numbers***
Bacteria	*Salmonella enterica* subsp. *enterica* serovar Typhi	Thyphoid fever	Human faeces	0.2-8.0
	Salmonella enterica subsp. *enterica* serovar Paratyphi	Paratyphoid fever	Human faeces	
	Salmonella enterica subsp. *enterica* serovar Enteritidis and *Salmonella enterica* subsp. *enterica* serovar Typhimurium	Salmonellosis/ gastroenteritis	Human/animal	

	Shigella sp. (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, *Shigella sonnei*)	Dysentery	Human faeces	0.1-1.0
	Vibrio cholera	Cholera	Human/animal	
	Vibrio parahaemolyticus	Gastroenteritis	Human faeces	10^6-10^7
	Escherichia coli (*E. coli* O:148; O:157; O:124)	Gastroenteritis	Human/animal	10^4-10^5
	Campylobacter sp.	Gastroenteritis	Human/animal	6- 8 x 10^4
	Clostridium perfringens		Human/animal	
	Faecal streptococci Enterococci		Human/animal	4.7 x 10^3 - 4 x 10^5
Viruses	Poliovirus	Poliomyelitis	Human faeces	180 – 500,000
	Rotavirus	Diarrhoea, vomiting	Human faeces	400 – 85, 000
	Adenovirus	Gastroenteritis	Human faeces	
	Norwalk virus	Diarrhoea, vomiting	Human faeces	
	Hepatitis A virus	Hepatitis	Human faeces	
Protozoa	*Cryptosporidium parvum*	Diahorrea		0.1 – 39.0
	Entamoeba histolytica	Amoeba dysentery		0.4
	Giardia lamblia cysts	Diahorrea		12.5 – 20,000

*Note: Number of infectious particles in raw sewage per mL (Adapted from Grabow, 2001; Ashbolt, 2004)

Viruses are among the most important, and potentially most hazardous of the pathogens found in wastewater (Verbyla *et al.* 2015). Viruses are of particular concern when present in wastewater due to their characteristically low (<10) infectious dosages (Haas *et al.* 1999; Murray *et al.* 2001). Untreated

wastewater can contain a range of viruses which are pathogenic to humans (Verbyla *et al.* 2015). Viruses are commonly more resistant to treatment processes and requires smaller doses to cause infection when compared with other pathogen types (Verbyla *et al.* 2015). More than 100 known species of viruses are excreted in human waste, and some of them are notably resistant to wastewater treatment (Verbyla *et al.* 2015). The most generally detected pathogenic viruses in wastewater are the enteroviruses, such as: poliovirus types 1 and 2, echovirus, enterovirus and coxsackievirus (Verbyla *et al.* 2015). Enterovirus outbreaks have occurred because of recreational water becoming contaminated with human faecal waste (Hewitt *et al.* 2011). Most of the enterovirus infections are asymptomatic or only cause mild illnesses, such as mild upper respiratory tract infections or non-specific febrile illness (Okoh *et al.* 2010). Withal, enteroviruses can also induce an extensive variety of clinical illnesses including acute haemorrhagic conjunctivitis, undifferentiated rash, aseptic meningitis, acute flaccid paralysis, myocarditis and neonatal sepsis-like disease (Okoh *et al.* 2010). Recently was reported that members of enterovirus-B are the most frequently to appear in wastewater, followed by enterovirus--A and then enterovirus-C, members of enterovirus-D are rarely reported in municipal wastewater (Brinkman *et al.* 2017). Other viruses which have been detected in wastewaters include adenoviruses, rotaviruses, reoviruses, astroviruses, and caliciviruses (Brinkman *et al.* 2017). Like the enteroviruses, the viruses cause a range of infections including acute gastroenteritis, respiratory tract infections, diarrhea, pneumonia, and conjunctivitis (Brinkman *et al.* 2017). The rotaviruses are the most severe of all the enteric viruses. Symonds *et al.* (2009) discovered adenoviruses, enteroviruses, noroviruses, and picobirnaviruses in treated wastewater in 12 cities of the United States.

1.4) CONVENTIONAL TREATMENT

The main objective of wastewater treatment is allowing human and industrial effluents to be disposed without danger to human health or unacceptable damage to the natural environment (Bartolomeu *et al.* 2018). The conventional wastewater treatment processes are designed to reduce solids in suspension, biodegradable organic products, microorganisms and nutrients (Bartolomeu *et al.* 2018). Conventional wastewater treatment consists of a combination of physical, chemical, and biological processes and operations to remove solids, organic matter and, sometimes, nutrients from wastewater (Bartolomeu *et al.* 2018). The wastewater treatment process can be divided into four main stages, specifically, the preliminary, primary, secondary and tertiary treatments (Naidoo *et al.* 2014; Bartolomeu *et al.* 2018). In some countries, disinfection to remove pathogens sometimes follows the last treatment step.

In general, throughout the world, wastewater from urban areas are secondarily, rarely tertiarily treated and released into the seawater far from beach areas (Macauley *et al.* 2006). Although the secondary effluent contains high concentrations of microorganisms, the effect of water dilution makes it acceptable in terms of quality indicators (Macauley *et al.* 2006). However, the changing patterns of

infectious diseases and the emergence of multidrug-resistant bacteria introduce serious risk to the discharge of wastewater not properly treated into the environment (Macauley *et al.* 2006). The traditional tertiary treatments used for reducing the microbial load are toxic to the aquatic species, induce genetic damage of several microorganisms and are expensive (Macauley *et al.* 2006) and consequently, development of new technologies for wastewater disinfection must be considered.

- **Preliminary treatment**

Preliminary treatment use screens to remove larger debris such as paper, plastic or any other foreign material which may damage downstream plant equipment (Naidoo *et al.* 2014). This is followed by further removal of grit and silt (Naidoo *et al.* 2014). Removal of these materials is necessary to enhance the operation and maintenance of subsequent treatment units (Naidoo *et al.* 2014).

- **Primary treatment**

Primary treatment aims to reduce any settleable solids, as well as oils, grease, fats, sand and grit within the wastewater via settling and sedimentation processes (Sonune *et al.* 2004). The steps involved in primary treatment are entirely mechanical and by means of filtration and sedimentation (Sonune *et al.* 2004)**.** Around 50-70% of the total suspended solids, 65% of the grease and oil, 25 - 50% of the incoming biochemical oxygen demand are removed throughout primary treatment (Sonune *et al.* 2004). Some organic phosphorus, organic nitrogen, and heavy metals associated with solids are removed during primary sedimentation, however, colloidal and dissolved constituents are not affected (Sonune *et al.* 2004). It is utilized clarifiers or settling tanks, which take away the settleable inorganic solids and the settleable organics from the wastewater (Sonune *et al.* 2004). The effluent from primary sedimentation units is designed as primary effluent (Sonune *et al.* 2004). In many industrialized countries, primary treatment is the minimum level of preapplication treatment required for wastewater irrigation (Sonune *et al.* 2004). It may be considered sufficient treatment if the wastewater is used to irrigate crops that are not consumed by humans or to irrigate orchards, vineyards, and some processed food crops (Faurès *et al.* 2012).

- **Secondary treatment**

The aim of secondary treatment is the further treatment of the effluent from primary treatment to remove the residual organics and suspended solids (Sonune *et al.* 2004). This step results in organic matter removal of approximately 90% (EPA, 1997). Typically, secondary treatment involves the removal of biodegradable dissolved and colloidal organic matter using aerobic biological treatment processes (Sonune *et al.* 2004). Aerobic biological treatment is performed in the presence of oxygen by aerobic microorganisms (principally bacteria) that metabolize the organic matter in the wastewater, thereby producing more microorganisms and inorganic end-products (principally CO_2, NH_3, and H_2O) (Faurès

et al. 2012). Numerous aerobic biological processes are used for secondary treatment diverging primarily in the method in which oxygen is provided to the microorganisms and in the rate at which organisms metabolize the organic matter (Sonune *et al.* 2004). This stage happens in biological reactors and is based on the biochemical degradation that naturally occurs in natural water environments, such as lakes and rivers (Cakir *et al.* 2005; WWAP, 2017). It is required to insert energy into the system to sustain the oxygen supplement, to allow the conversion of the organic matter into degradation products as sludge (biomass) and carbon dioxide (Cakir *et al.* 2005; Corcoran *et al.* 2010; Bartolomeu *et al.* 2017). The aerobic degradation process avoids the anaerobic process, in which the formation of methane will take place (greenhouse gas) (Corcoran *et al.* 2010). At this point, if the wastewater parameters are below the legally imposed limits the wastewater effluents are considered good enough to be released into the environment (Directive nº 113/2012), specified the limiter values of *E. coli* to 1800 CFU/100 mL and 1200 CFU/100 mL and of *Enterococci* to 660 CFU/100 mL and 350 CFU/100 mL for transitional bathing waters or interior bathing waters and coastal water (Cakir *et al.* 2005; Bartolomeu *et al.* 2017).

- **Tertiary treatment**

The use of the tertiary treatment is not very frequent worldwide (Rosal *et al.* 2010; Wang *et al.* 2016; Bourgin *et al.* 2017). The urban wastewater treatment-related European Commission Directive (Directive 91/271/EEC) points to the mandatory collection and secondary treatment application to wastewater in urban settlements (Rosal *et al.* 2010; Wang *et al.* 2016; Bourgin *et al.* 2017). The application of more advanced treatments is, however, mandatory when the release of wastewater takes place in sensitive areas (Corcoran *et al.* 2010; Chamy *et al.* 2013) where the previous applied treatments were not adequate to keep the levels as indicated by the quality guidelines.

The tertiary and /or advanced wastewater treatment generally follows secondary treatment and aids the removal of those wastewater constituents and pathogenic microorganisms (*e.g.* faecal coliforms, *Salmonella* sp. and enteric viruses) that are not removed by previous treatments (EPA, 1997). The tertiary treatment may be divided into three main treatment types namely: chemical, physical and irradiation (Sonune *et al.* 2004). Physical treatments generally involve one or a combination of treatments such as rapid sand filtration, additional nutrient removal or carbon adsorption which is employed prior to chlorination to remove any remaining suspended solids as well as reduce the number of nitrates, phosphates and soluble organic matter present (SOPAC, 1999). Following this, chemical and irradiation disinfection may occur, and generally involves one or a combination of treatments involving chlorination and ultraviolet light exposure or ozonation (Table 2), the choice of which depends solely on the incoming effluent quality, ease and cost of installation, maintenance and operation as well as effects on flora, fauna and recreational users from final effluent re-use and disposal into respective receiving water bodies (US EPA, 1999). Chlorination is the most common method of ensuring microbiological safety in tertiary effluents since it effectively inactivates bacteria and viruses (Basu *et al.* 2007). However, its massive utilization may lead to the formation of disinfection byproducts with potential health hazards (Basu *et al.* 2007). In fact, one of the major disadvantages associated with chlorination

is the production of toxic by products such as trichloromethanes and other chloramines which cause severe harmful effects on the receiving water bodies into which they are discharged (Basu *et al.* 2007).

Table 2. Disinfection by chemical and irradiation treatment (Adapted from Naidoo *et al.* 2014).

Disinfection Treatment	Method	Advantages	Disadvantages
Chlorination	Chlorine gas is a strong oxidant, which reacts with any form of organic matter. Elemental chlorine comes into contact with water, and it is hydrolysed to hypochlorous acid (HOCl) and hypochlorite (-OCl)	More cost effective than other methods Accurate and easy application	Extremely unstable and deteriorates quickly Production of toxic by-products (trichloromethanes, chloramines) Harmful effects on the receiving water bodies
Ultraviolet Light	Electromagnetic energy from a mercury arc lamp to irradiate and disinfect wastewater effluent. UV light penetrates the cell wall damaging their genetic material and avoiding survival	Decreased environmental toxicity Eliminates the need to handle/transport/store corrosive/toxic chemicals Generation of disinfectant by products is highly reduced	When UV is applied at lower doses, microorganisms tend to reverse the damage through their own cellular repair mechanisms
Ozonation	Must often be generated onsite by the passage of oxygen through a high voltage electric field, reacting with any organic matter present within the wastewater	Elimination of any odours Does not result in any residual compounds Easily produced from air	High costs involved Totally dependent on the available power source

The wastewater treatment processes only eradicate between 50% and 90% of viruses present in wastewater, permitting for a significant viral load to be released in effluent discharge (Karmakar *et al.* 2008; Okoh *et al.* 2010). The range of enteric virus reduction varies according to the sewage treatment system used and the virus type (Okoh *et al.* 2010). Okoh *et al.* (2010) demonstrated that enteric viruses are inherently more resistant to common disinfectants than bacterial indicators. These authors observed that bacterial indicators, *E. coli* and *Enterococcus faecalis*, were rapidly inactivated by chlorine with inactivation levels of (>5 $\log_{10}$ units) while there was poor inactivation (0.2 to 1.0 $\log_{10}$ unit) of F^+ -specific RNA (FRNA) bacteriophage (MS2) at doses of 8, 16, and 30 mg/L of free chlorine (Okoh *et al.* 2010). It

was also showed that the inactivation levels of naturally occurring coliphages were significantly lower than that of coliforms after chlorination (Okoh *et al.* 2010).

Although the transmission of viruses has been reduced by the development of good water supplies and hygienic procedures for a whole range of human activities, it is still important to find novel, convenient, environmentally-friendly and inexpensive methods to avoid microbial contamination (Naidoo *et al.* 2014). Currently, photodynamic therapy (PDT) is receiving considerable interest as a potential antimicrobial treatment (Alves *et al.* 2015). PSs, namely porphyrin derivatives, are promising chemical disinfectants for the inactivation of pathogens as they are effective in the presence of light and oxygen, without the formation of potentially toxic products (Alves *et al.* 2015).

1.5) PHOTODYNAMIC THERAPY

Photodynamic inactivation (PDI) is defined as the process of cell destruction by oxidative stress resulting from the interaction between light and a PS, in the presence of molecular oxygen (Alves *et al.* 2015). PDI of bacteria, viruses and fungi has been extensively studied in recent years, proving to be a promising alternative to conventional methods. In the last years, scientific research in this area has gained importance due to great developments in the field of materials chemistry but also because of the serious problem of the increasing number of bacterial species resistant to common antibiotics (Tavares *et al.* 2010). The applicability of PDI goes far beyond the clinical field (Alves *et al.* 2015). Due to its multi-target nature, and therefore low probability of triggering the development of resistance in microorganisms (Lauro *et al.* 2002; Pedigo *et al.* 2009; Tavares *et al.* 2010; Costa *et al.* 2011), this therapy has been tested in various research areas as an alternative approach to actual methods to control water quality in different environmental (*e.g.* aquaculture, hospital wastewater), microbiological food quality; and also in the disinfection and sterilization of materials and surfaces (Alves *et al.* 2015)

1.5.1) PHOTODYNAMIC THERAPY PRINCIPLES

The use of light in the treatment of diseases, designed as phototherapy, has been used since antiquity (Ackroyd *et al.* 2001). In the beginning of the twentieth century, the photodynamic effect concept was defined (Ackroyd *et al.* 2001) as the use of a light (artificial light source or sunlight), molecular oxygen, O_2 (dissolved in the reaction medium) and an intermediate agent (PS) which has the capacity to absorb and transfer energy from light to molecular oxygen, allowing the formation of highly cytotoxic species, reactive oxygen species (ROS), that cause damage to living tissue, or even destruction: singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2), superoxide anion radical ($O2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$) (Henderson and Dougherty, 1992; Bonnett, 2000; Alves *et al.* 2008). In PDI, the

PS adheres to the microorganisms, proceeded by an irradiation process with light with adequate wavelength (λ), especially coincident with one of the maximum absorption peaks of the used PS (Calin and Parasca, 2009). Throughout the irradiation process, ROS will be generated, that will oxidize several cellular components such as lipids and proteins (Alves *et al.* 2013). Oxidative reactions on these components conduct to changes in their structure and consequently loss of function (Alves *et al.* 2013). The oxidative damages created by ROS occurs on the external and internal structure of microorganisms, leading to the oxidation of lipids, proteins, degradation of essential enzymes, and damage of the nucleic acids (DNA/RNA) that cause morphological changes and disrupt its functionality (Alves *et al.* 2014; Almeida *et al.* 2015; Wainwright *et al.* 2016; Vieira *et al.* 2018). Specifically, in viral PDI, ROS must bind precisely to vital components, like lipid envelope (when present), nucleic acids or to the protein coat (Vieira *et al.* 2018).

ROS have a very short life time owing to their unstable electronic configuration (Alves *et al.* 2014). Oxygen singlet has a lifetime of 3 – 4 μs and its diffusion range relies on the nearby medium, being less than 50 nm in a protein-rich lipid layers ambient (Alves *et al.* 2014). The PDI efficiency greatly depends on the PS localization through the irradiation process, since the nearness of the PS to its potential targets is essential (Alves *et al.* 2014).

1.5.2) MECHANISM OF ACTION

In the photodynamic process, the PS absorbs energy from the light and transfers to molecular oxygen (Hamblin, 2008). The absorption of a photon at an appropriate wavelength, initially generates the production of an unstable electronically excited state of the PS molecule, with a very short lifetime (Hamblin, 2008). Then, the excited PS molecule can decay to the ground state by releasing light emission, fluorescence (radiative pathway) or by intersystem crossing that allows the excitation for a triplet state which leads to a vibrational relaxation (Hamblin, 2008). In the triple state molecules have a longer lifetime (Hamblin, 2008). At this moment, the PS can return to an excited state by spin inversion and phosphorescence, or by a non-radiative process (Hamblin, 2008). Once the triple state promotes a longer lifetime, PS molecule can pursue two different reactions (Type I and Type II) (Hamblin, 2008).

The type I mechanism (Figure 1) implicates hydrogen-atom abstraction or electron-transfer between the excited PS and a substrate, producing free radicals (Costa *et al.* 2012). These radicals can react with oxygen to form active oxygen species, such as the superoxide radical anion (Costa *et al.* 2012). Superoxide is not particularly reactive in biological systems still, when protonated, can lead to the production of hydrogen peroxide and oxygen or highly reactive hydroxyl radicals (Costa *et al.* 2012).

Whereas type II photooxidation mechanism (Figure 1) is considerably less complex mechanistically than type I and usually there are far fewer products (Costa *et al.* 2012). In this pathway, the excited triplet state PS ($^3PS^*$) can transfer the excess energy to molecular oxygen (O_2) and relax to

its ground state (^{1}PS) forming an excited singlet molecular oxygen ($^1O_2$) (Costa *et al.* 2012). $^1O_2$ is highly electrophilic, thus, it conducts to the inhibition of protein synthesis and molecular alteration of DNA strands, which modifies the transcription of the genetic material during its replication and, consequently, leading to microbial death (Bonnet, 2000). Like nucleic acids and proteins, unsaturated lipids are also very prominent targets of $^1O_2$ and free radical attack (Costa *et al.* 2012). Lipid peroxidation-ensuing reactions can alter surrounding proteins, nucleic acids and other molecules, in addition to the lipids themselves (Käsermann *et al.* 1998; Costa *et al.* 2012).

Both type I and type II mechanisms may occur simultaneously or solely, and the ratio between these processes depends on the PS used and on the concentrations of substrate and oxygen (Bartolomeu *et al.* 2018). The competition between organic substrates and molecular oxygen for the ^{3}PS* determines whether the reaction pathway is type I or type II and the major mechanism can be changed during the development of the PDI process (Bartolomeu *et al.* 2018).

Type I mechanism

$PS + h\,v \rightarrow PS^*$

$PS^* + Subs \rightarrow PS^{\bullet-} + Subs^{\bullet+}$ *or* $PS^* + Subs \rightarrow PS^{\bullet+} + Subs^{\bullet-}$

$PS^{\bullet-} + O_2 \rightarrow PS + O_2^{\bullet-}$

$PS^* + O_2 \rightarrow PS^{\bullet+} + O_2^{\bullet-}$

$PS^* + R\text{-}H \rightarrow PS\text{-}H^{\bullet} + {}^{\bullet}R$

${}^{\bullet}R + O_2 \rightarrow {}^{\bullet}OOR$

${}^{\bullet}OOR + R\text{-}H \rightarrow RO_2H + R^{\bullet}$

Type II mechanism

$^3PS + O_2 \rightarrow {}^1PS + {}^1O_2$

$^1O_2 + Subs \rightarrow$ *Substrate oxidation*

Figure 1) Photodynamic therapy type I and type II mechanisms (Bartolomeu *et al.* 2018).

1.5.3) PHOTOSENSITIZERS

The first PS utilized in cancer photodynamic therapy (PDT) are mostly derivatives of hematoporphyrin, which is a synthetic porphyrin synthesized from heme (Ackroyd *et al.* 2001). PS evolution resulted mostly from the clinical necessity to upgrade their action and to diminish side effects (Ackroyd *et al.* 2001). Since it was detected substantial side effects in the tissues, it was required to develop new PS which absorb at higher wavelengths with the purpose of reaching the deepest parts of

the tissue for better PDT efficiency and selective localization (Ackroyd *et al.* 2001). Several of these new compounds are meso-tetra(hydroxyphenyl) porphyrins, phthalocyanines, 5-aminolevulinic acid (ALA, a prodrug), *N*-aspartyl chlorin e6, texaphyrins, tin ethyl etiopurpurin and benzoporphyrin derivative monoacid ring A (Ackroyd *et al.* 2001).

Besides dose and intensity of light, the efficacy of PDT also relies on the chemical structure of PS, binding ability and *in vitro* studies of target cells (Alves *et al.* 2014). The key element of their chemical structure is the porphyrin ring formed from four pyrrole molecules connected by methine bridges =CH– (Wainwright *et al.* 2016). Depending on the modification of the ring by the lateral substituents, porphyrin derivatives exhibit different spectroscopic properties (Wainwright *et al.* 2016). All these compounds have propensity to aggregate, what influences their physicochemical and spectroscopic properties (Wainwright *et al.* 2016). Since they are very effective chromophores of visible light, these compounds can produce free radicals and singlet oxygen (Kempa *et al.* 2015). Due to these exclusive properties, porphyrins have been efficaciously used for photodynamic therapy (PDT) (Kempa *et al.* 2015). It is fundamental for the development of this therapy to comprehend how PS function in microorganisms so it can be possible to create more suitable PS molecules (Alves *et al.* 2014).

It is already known that PS molecular structure is a vital factor for the achievement of PDT, once it uses the energy of radiation to yield useful energy (Alves *et al.* 2014). Therefore, some ideal properties of these compounds can be emphasized (Almeida *et al.* 2011; Alves *et al.* 2014):

- Photostability;
- High chemical purity and easy synthesis;
- Positive charge and symmetry;
- Nontoxic in absence of light;
- Soluble (should not precipitate or aggregate in biological systems);
- High yield production of ROS;
- Amphiphilic molecules to enable its penetration and distribution;
- Be easy to transport into the specific infection site;
- Not require high cost for its activation;
- High absorption peak within the visible range;
- Wide spectrum of action;
- Non-mutagenic and non-genotoxic;
- Selectivity for the target cells.

Hence, a PS can be conceived in agreement with certain biological parameters or to produce determined ROS with the objective to turn the process more appropriate, mainly to reach more easily the microorganisms (Wainwright, 1998). The kinetics of a PS lean on its positive charge (cationic) and water solubility (Wainwright, 1998). The PS toxicity is related to its chemical properties, concentration, formulation, microenvironment of activity (Wainwright, 1998). In addition, the PS molecule can be

directed to certain membrane enzymes or even to specific cellular compartments within the microorganism (Wainwright, 1998). Many PS can be easily formulated by partial syntheses from plenty natural materials, like heme, chlorophyll and bacteriochlorophyll (Wainwright, 1998). This feature leads to economical but also environmental advantages compared to intricate total chemical synthesis (Wainwright, 1998).

The majority of PS are porphyrins and chlorins, these can be applied as free-bases or matched with a metal in the inner core of the macrocycle; the structure of these macrocycles is analogous to protoporphyrin IX, the ligand of the heme group and to chlorophylls (Josefsen *et al.* 2008). The PS are usually labeled as first, second or third generation (Josefsen *et al.* 2008). The first generation is associated to derivatives of hematoporphyrin (HpD), specifically Photofrin, the first sensitizing drug molecules used in PDT (Josefsen *et al.* 2008). Moreover, this elaborate mixture of porphyrins has been extensively used to treat cancers on investigational basis (Josefsen *et al.* 2008). Two decades ago appeared the designated second generation of PS in order to surpass some limitations of the first generation (Josefsen *et al.* 2008; Luksiene *et al.* 2009). The crucial objective was to acquire pure compounds with improved photophysical properties (Josefsen *et al.* 2008). Some of this PSs are replaced in the *meso* positions (5, 10, 15 and 20) with phenyl groups carrying halogens or other bulky groups (Josefsen *et al.* 2008; Luksiene *et al.* 2009). The majority absorb light at the longer wavelengths and display maximum absorption peak in the red wavelength, leading to its application in lower concentrations (Josefsen *et al.* 2008; Luksiene *et al.* 2009). Generally, the efficiency of producing $^1O_2$ is followed by high stability and no toxicity in the absence of light (Josefsen *et al.* 2008). Afterwards a third-generation of PS has been created in order to increase and direct the PS affinity to precise targets (Josefsen *et al.* 2008).

1.5.4) ADVANTAGES AND LIMITATIONS OF APDT

The success of PDT is directly correlated with the capacity of the PS to generate free radicals (type I mechanism) through the photodynamic process and/or generate $^1O_2$ (type II mechanism) (Almeida *et al.* 2011; Alves *et al.* 2014). For that reason, the kind of PS (monomers, dimers or higher order aggregates) is a significant factor for the achievement of PDT, since it guarantees superior efficiency in the photoinactivation. *In situ* and also *in vivo* assays demonstrate that when micromolar concentrations are utilized, negative effects of PS on microorganisms do not happen, however, these concentrations are efficient enough to induce microbial inactivation (Almeida *et al.* 2011; Alves *et al.* 2014). Moreover, the recovery and further reutilization of the sensitizer molecules is a relevant factor, owing to the fact that the process becomes easy to apply, low-cost and environmental-friendly (Almeida *et al.* 2011; Alves *et al.* 2014).

There are some aPDT benefits that deserve to be emphasized, such as (Almeida *et al.* 2011; Alves *et al.* 2014):

- The opportunity of being applied to a widespread variety of microorganisms;
- To have a small period of inactivation, in which no photo-resistance or mutagenicity is established, even after several treatments;
- To be an effective antimicrobial treatment where the effectiveness is independent of the antibiotic resistance pattern.

Allegedly, cytotoxicity and side effects of PDT should principally be associated with the distribution and amount of the PS, the oxygen availability and intensity of light (Almeida *et al.* 2011; Alves *et al.* 2014). Still, one of the restraints lies on the fact that it is a localized process and for that reason, the treatment can merely be utilized in surface areas (Almeida *et al.* 2011; Alves *et al.* 2014). In addition, it was discovered that the microbial photoinactivation is higher in the absence of organic matter, yet this problem can be conquered by rising adequately the PS concentrations (Almeida *et al.* 2011; Alves *et al.* 2014).

The applications studied and suggested in the latest years and the progress created in this research area prove that photoinactivation is an auspicious method of sterilization/disinfection with prosperous practical application in a short period (Alves *et al.* 2015).

1.5.5) PHOTODYNAMIC THERAPY INACTIVATION OF PATHOGENIC MICROORGANISMS

aPDT has revealed to be a very auspicious alternative and an effective technique to inactivate microorganisms (bacteria, including multidrug-resistant strains, viruses, microfungi, protozoa and yeasts), besides being environmentally friendly (Alves *et al.* 2014). Moreover, it is also effective in inactivating biofilms (complex and highly microbial structured matrix) and spores (Alves *et al.* 2014). With this approach, microbial infections can be contained and, until now, the development of resistance was not noticed (Alves *et al.* 2014).

The Gram-positive and Gram-negative bacteria are not equally affected by aPDT (Jori *et al.* 2004). Gram-positive are normally more susceptible to aPDT than Gram-negative, owe to the difficulties of the PS to enter in Gram-negative cells (Jori *et al.* 2004). This happens due to the physiological and structural differences of their cell wall (Jori *et al.* 2004). The Gram-negative cells have an external protection composed by a lipopolysaccharide layer strongly charged that obstructs the penetration of $^1O_2$ (Jori *et al.* 2004). Therefore, what varies between both bacteria is the way that the PS penetrate the membrane (Jori *et al.* 2004).

Even though fungi are more complex targets than bacteria, the behavior of such cells to photodynamic processes is less controlled by structural factors as compared with bacteria (Pereira *et al.* 2012). Pereira *et al.* (2012) observed that the *Candida* cells can be extensively killed by anionic PSs (Pereira *et al.* 2012). Moreover, fungal cells can be destroyed at photodynamic low dose rates which makes possible a 'therapeutic window' (Pereira *et al.* 2012). The photodynamic mechanism damages

fungal cells by ROS bursting cell walls and membranes, consequently permitting the PS to be translocated into the cell (Pereira *et al.* 2012). Once inside the cell, oxidizing species created by light excitation promote photodamage to internal cell organelles and lastly cell death (Pereira *et al.* 2012). Multiple cellular targets are disposable for the photo-oxidative effect caused by singlet oxygen including inactivation of enzymes and other proteins and peroxidation of lipids, inducing lyses of cell membranes, mitochondria and lysosomes (Pereira *et al.* 2012).

The sensitivity of viruses to photodynamic procedures was reported in the 1930s (Schultz *et al.* 1928; Perdrau *et al.* 1933) but only within the last 30 years, with the development of new PS, and an increment of light technologies (*e.g* lasers and LED), the photodynamics techniques for inactivation of viruses received more attention (Käsermann *et al.* 1998). Many of the initial clinical studies of PDT for treatment of infections were directed towards viral lesions (Kharkwal *et al.* 2011). Topical application of PDT was commonly tested in the treatment of herpes simplex lesions (Kharkwal *et al.* 2011). Herpes keratitis was treated by proflavine photodynamic viral inactivation (Moore *et al.* 1972). Since then, a great variety of viruses has been inactivated by photodynamic treatment *in vitro* conditions (Almeida *et al.* 2011). In clinical, the procedures are limited to the treatment of papillomatosis, caused by human papillomatosis virus (HPV), like laryngeal papillomatosis (Mullooly *et al.* 1990) and epidermodysplasia verruciformis (Karrer *et al.* 1999) and, in a small scale, to the treatment of viral complications in AIDS patients (Lavie *et al.* 1995; Smetana *et al.* 1997). However, in last years, considerable progress has been made in the viral photodynamic disinfection of blood products. The viral contamination in blood and blood products is associated, commonly, the immunodeficiency viruses (HIV) (Sloand *et al.* 1995), hepatitis viruses (Mannucci, 1992; Klein, 1994; Sloand *et al.* 1995), cytomegalovirus (Klein, 1994), human parvovirus B19 (Azzi *et al.* 1993) and human T-cell lymphotropic virus type I and type II (Klein, 1994). The photoinactivation of hepatitis viruses in blood products has also been successfully tested against the hepatitis C virus (HCV) (North *et al.* 1992; Müller-Breitkreutz *et al.* 1998; Vanyur *et al.* 2003; Cheng *et al.* 2010), hepatitis B virus (HBV) (Lin *et al.* 2008) and hepatitis A virus (HAV) (Casteel *et al.* 2004). Inactivation of cytomegalovirus (O'Brien *et al.* 1992) human parvovirus B19 (Mohr *et al.* 1997) and human T-cell lymphotropic virus (Sieber *et al.* 1987) in blood products was also efficiently achieved after photodynamic treatment.

The existence of a simple and quantitative assay to follow the viral photoinactivation process is important (Costa *et al.* 2012). Traditional viral quantification techniques, such as *in vitro* viral cultures, are time-consuming and labor-intensive processes (Costa *et al.* 2012). Molecular quantitative methods such as nucleic acid amplification procedures, including real time PCR, are rapid and sensitive but detect only viral nucleic acid and do not determine infectivity (Costa *et al.* 2012). When the virucide properties of different photosensitizing compounds are initially evaluated, bacteriophages (or phages) can be useful as surrogates of mammalian viruses (Costa *et al.* 2012). The use of bacteriophages in an initial screening have some advantages: (i) the detection methods are much simpler, faster and cheaper than those of mammalian viruses, avoiding the advanced facilities and equipment needed for propagating human pathogens; (ii) bacteriophages are non-pathogenic to humans; (iii) bacteriophages can be grown

to higher concentrations than most mammalian viruses (Costa *et al.* 2012). In addition, the enveloped viruses are more sensitive to PDT that the non-enveloped viruses (Rywkin *et al.* 1994; Käsermann *et al.* 1998). As most of the bacteriophages are non-enveloped, they are more difficult to suffer photoinactivation than the enveloped viruses, as demonstrated by Costa *et al.* (2012). A PDI protocol that is effective to inactivate a non-enveloped bacteriophage will most likely be effective against enveloped mammalian viruses. Several bacteriophages were used in photoinactivation studies, in phosphate buffered saline solution (PBS) and wastewater, as surrogates for virus models (Table 3): MS2 (Casteel *et al.* 2004), M13 (Abe *et al.* 1997), PM2 (Specht *et al.* 1994), Qβ (Lee *et al.* 1997), PRD1 (Hotze *et al.* 2009), λ (Martin *et al.* 2005), φ6 (Wagner *et al.* 1998), R17 (Wagner *et al.* 1998), T7 (Hotze *et al.* 2009) and T4-like (Costa *et al.* 2011), and the results show that the photodynamic therapy can be efficient methodology to inactivate virus. The bacteriophage T4 is one of the most extensively viruses studied in the last 25 years (Karam *et al.* 2010). Its popularity among researchers is related to how easily this bacteriophage and some of its relatives can be propagated in widely available nonpathogenic laboratory strains of *E. coli* and the diversity of experimental approaches that can be used to analyze its DNA genome and the RNA and protein products it encodes (Karam *et al.* 2010).

Table 3. aPDT of bacteriophages (Costa *et al.* 2012).

Photosensitizers	Bacteriophages	Inactivation (PFU/mL)	Reference
Glycoconjugated meso-tetraarylporphyrins	T7	<3 log	Gábor *et al.* 2001
	T7	<3.5 log	Egyeki *et al.* 2003
Tetrasulfonated meso-tetraarylporphyrin derivatives	MS2	>3.8 log	Casteel *et al.* 2004
meso-Tetrakis(1-methylpyridinium-4-yl)porphyrin	λ	<7 log	Kasturi *et al.* 1992
	MS2	>4.1 log	Casteel *et al.* 2004
	T4	7 log	Costa *et al.* 2008; Costa *et al.* 2010
	T7	<4 log	Zupán *et al.* 2008
5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	T4	7 log	Costa *et al.* 2008; Costa *et al.* 2011
5-(4-methoxicarbonylphenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	T4	7 log	Costa *et al.* 2008
5-(4-carboxyphenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin	T4	3.9 log	Costa *et al.* 2008
5,10-bis(4-carboxyphenyl)-15,20-bis(1-methylpyridinium-4-yl)porphyrin	T4	1.4 log	Costa *et al.* 2008

5,15-bis(4-carboxyphenyl)-10,20-bis(1-methylpyridinium-4-yl)porphyrin	T4	1.2 log	Costa *et al.* 2008
5,10,15-tris(1-methylpyridinium-4-yl)-20-phenylporphyrin	T7	1.7 log	Zupán *et al.* 2008
Methylene blue	M13	2.2 log	Abe *et al.* 1995; Abe *et al.* 1997
	Qβ	7–8 log	Lee *et al.* 1997; Schneider *et al.* 1998
Phenothiazine derivatives	R17	4–7 log	Wagner *et al.* 1998
	φ6	4–6.5 log	Wagner *et al.* 1998
Rose Bengal	PRD1	~3.5 log	Hotze *et al.* 2009
	T7	~4.5 log	Hotze *et al.* 2009
Riboflavin	λ	<4 log	Martin *et al.* 2005
Polyhydroxylated fullerene	MS2	~4 log	Badireddy *et al.* 2007
	PRD1	~2.5 log	Hotze *et al.* 2009
	T7	~3.5 log	Hotze *et al.* 2009
	MS2	~5 log	Hotze *et al.* 2009
Form	T4	~8 log	Vieira *et al.* 2019

The location and binding site of the PS is highly dependent on the structure and intramolecular charge distribution and an important factor in microbial PDI (Merchat *et al.* 1996; Costa *et al.* 2012). Usually, positively charged PS are more effective and can function at lower concentrations in comparison to anionic and neutral PS molecules (Demidova *et al.* 2005; Costa *et al.* 2012). The positive charges on the PS molecule seem to create a steady electrostatic interaction between negatively charged sites at the viral capsids/envelopes and the positively charged PS. This interaction draws the PS onto these steady electrostatic sites that are crucial for the metabolism and stability of a specific microorganism, which will be consequently affected by ROS (Dowd *et al.* 1998; Costa *et al.* 2012). This type of association augments the efficacy of the photoinactivation process. Cationic PS photodamage can be prompt into the viral outer structures or nucleic acid by PS localized in its surrounding or by PS binding (Wainwright *et al.* 2004; Costa *et al.* 2012). For example, it is more probable that positively charged PS will be efficacious in producing nucleic acid damage than anionic or neutral congeners, which will functionate mostly against the outer side of the microorganism (Wainwright *et al.* 2004; Costa *et al.* 2012). The toxicity of a PS can be adjusted by the insertion of selected substituents on the macrocycle periphery (Casteel *et al.* 2004; Costa *et al.* 2012). Therefore, the physicochemical properties of a synthetic PS can be managed to improve its interactions with the structural properties of the viruses, like viral capsids (Casteel *et al.* 2004; Costa *et al.* 2012). Besides the charge and toxicity, PS concentration is as well a significant parameter that must be considered since viral aPDT demonstrated to be highly affected by PS concentration (Costa *et al.* 2008; Costa *et al.* 2012). Enhancing the PS concentration, the time needed to achieve complete viral inactivation is reduced, consequently rising the efficiency of the aPDT technique (Costa *et al.* 2008; Costa *et al.* 2012).

1.5.6) MOLECULAR TARGETS OF APDT IN BACTERIOPHAGES

The short-lived ROS produced by photodynamic mechanisms are responsible for the damage caused in critical molecular targets such as the membrane lipid integrity (Costa *et al.* 2012). Different viral targets, such as the envelope, lipids and proteins, capsid, core proteins and the nucleic acid may be affected by singlet oxygen and/or other ROS (Wainwright, 2003; Costa *et al.* 2012), like hydrogen peroxide, superoxide and hydroxyl radicals, to complete the loss of infectivity. Exhaustive photophysical and photochemical studies of the interactions between ROS created by the PS and key biomolecules such as proteins, lipids and nucleic acids are indispensable for the knowledge and prediction of photosensitization process efficiency (Costa *et al.* 2012). Nevertheless, the studies performed express that the primary target of PDI depends on the chemical structure of the PS, the targeted virus and the mechanism of photoinactivation (Wainwright, 2003; Costa *et al.* 2012). For example, it was shown that methylene blue and aluminiumphthalocyanine-tetrasulphonate produce damages in the nucleoprotein complexes but not in the proteins in M13 bacteriophage and vesicular stomatitis virus (Wainwright, 2003).

Depending on the viruses, the nucleic acid can be either RNA or DNA, single or double stranded (Costa *et al.* 2012). The size of the nucleic acid also differs depending on the viruses. In the literature there is information which explain that both DNA and RNA bacteriophages are effectively inactivated by PDI (Garcia *et al.* 2009; Costa *et al.* 2012). There is substantial information that PS can bind to and penetrate viral membranes, to the point they intercalate with nucleic acids (Garcia *et al.* 2009; Costa *et al.* 2012). Upon activation by light, the generated ROS can cause the destruction of the nucleic acids, particularly at guanine residues, avoiding viral replication (Costa *et al.* 2012). Still, there is a difference in target selectivity depending on the mechanism involved: sugar moieties are usually attacked by radicals, generated via type I mechanism, while guanine residues are the targets of singlet oxygen, generated via type II mechanism (Costa *et al.* 2012).

From the four DNA bases, guanine is the most vulnerable component to suffer a type II photosensitization reaction, since it displays the lowest oxidation potential among DNA bases and it is the only base that can be oxidized by singlet oxygen (Wainwright, 2000; Costa *et al.* 2012). It is acknowledged that cationic porphyrins can bind to nucleic acids *via* intercalation into base pairs or self-stacking, inducing lesions upon photoinactivation due to the easy oxidation of guanine residues (Costa *et al.* 2012). The binding of cationic porphyrins to DNA is apparently owing to the electrostatic interaction among the positively charged substituents in the porphyrin macrocycle and the negatively charged phosphate oxygen atoms on DNA (Wainwright, 2000; Costa *et al.* 2012). However, the binding between porphyrin and DNA is not a requirement for an efficient photosensitization, because free porphyrins can be more effective in virus inactivation than the DNA-bound porphyrins (Costa *et al.* 2012). This observation, which conflicts with the commonly accepted idea that the porphyrin molecule must be in

close vicinity with the site of photosensitized damage, may be explained by the lower quantum yield of singlet oxygen by the bound porphyrin when compared with the free one (Wainwright, 2000; Costa *et al.* 2012;).

It was mentioned that enveloped viruses are inactivated more rapidly than non-enveloped viruses (Garcia *et al.* 2009; Costa *et al.* 2012), the damages caused by photodynamic reactions on unsaturated lipids present in their envelopes and/or on major envelope proteins, which act as PS binding-sites, modify their structure and avoid cell infection and virus replication (Costa *et al.* 2012). Though, some studies showed that non-enveloped viruses can also be efficiently inactivated by the action of PS (Kadish *et al.* 1967; Costa *et al.* 2012). The higher susceptibility of enveloped viruses to PDI, relatively to non-enveloped viruses, indicates that the viral envelope may be a more central target than nucleic acids for photosensitization, however, until today, no studies focus on the degradation of viral envelope lipids after PDI or on other viral internal lipids (Garcia *et al.* 2009; Costa *et al.* 2012). There are, though, many studies about the effects of PDI on viral envelope proteins as well as on other core proteins: the report about enveloped viruses being more easily inactivated than non-enveloped ones are based in indirect studies which relate the inactivation results of enveloped and non-enveloped viruses (Garcia *et al.* 2009; Costa *et al.* 2012). The enveloped viruses used in PDI protocols were only assayed for their protein alterations and no additional work was done concerning their lipids (Garcia *et al.* 2009; Costa *et al.* 2012).

Relative to proteins degradation by PDI, the results of different studies demonstrated that the main damage is the formation of protein cross-links, followed by other types of damage, which include loss of proteins, alterations in protein molecular conformation, mass and charge, and alterations in protein band intensity (Table 4) (Davies, 2003; Costa *et al.* 2012). When proteins are irradiated with UV or visible light in the presence of a PS, photooxidation of sensitive amino acid residues such as cysteine, L-histidine, methionine, tyrosine and tryptophan, and covalent cross-linking of peptide chains can be detected, conducting to the formation of molecular aggregates, dissolving their normal folding conformation, therefore forcing them into other conformations that affect their normal functioning (Davies, 2003; Costa *et al.* 2012). In fact, the formation of cross-linked/aggregated material seems to be a major consequence of photosensitized-mediated protein oxidation, and it has been confirmed that the formation of protein cross-links is not a primary photodynamic event, but a secondary reaction between the photooxidation products of sensitive amino acid residues and other groups in the protein (Costa *et al.* 2012). The PS combined with light can bring modifications in the folding of some enzymes, leading to the exposure of some amino acid residues usually shielded in the protein, and to the shielding of others frequently exposed in the molecule (Costa *et al.* 2012). These protein alterations lead to changes in properties such as solubility, absorbance, proteolytic susceptibility, and fluorescence emission of several of their amino acids (Verweij, 1982; Costa *et al.* 2012). These changes are mainly mediated by hydrogen peroxide and hydroxyl radical generation, although singlet oxygen mediated reactions may also occur (Costa *et al.* 2012). The amino acids positioned in the surface of the protein are photooxidized at a much faster rate than the residues hidden in the interior of the molecule (Costa

et al. 2012). If a protein is completely unfolded, susceptible amino acids may also be attacked and photodegraded (Costa *et al.* 2008).

Table 4. Degradation of viral structures after bacteriophages aPDT (Costa *et al.* 2012).

Bacteriophages	Photosensitizer	Damage	Reference
T7	Glycoconjugated meso-tetraarylporphyrins	Protein capsid; loosening of the protein-DNA interaction	Gábor *et al.* 2001
	Glycoconjugated meso-tetraarylporphyrins	Capsid and core proteins; loosening of protein-DNA interaction	Egyeki *et al.* 2003
	Meso-Tetrakis(1-methylpyridinium-4-yl)porphyrin	Capsid proteins; protein cross-links	Zupán *et al.* 2008
	Polyhydroxylated fullerene	Capsid proteins; protein cross-links	Hotze *et al.* 2009
M13	Methylene blue Aluminum phthalocyanine tetrasulfunate	Coat protein	Abe *et al.* 1995
PRD1	Polyhydroxylated fullerene	Capsid proteins; protein cross-links; phospholipids (less affected)	Hotze *et al.* 2009
Qβ	Methylene blue	Coat and maturation (A) proteins; formation of protein carbonyls; RNA-protein cross-links	Schneider *et al.* 1998
	Methylene blue	RNA-protein cross-links	Floyd *et al.* 2004
MS2	Polyhydroxylated fullerene	A protein	Hotze *et al.* 2009

1.5.7) POTENTIATION OF APDT BY POTASSIUM IODIDE (KI)

In last years, some studies have demonstrated that aPDT can be potentiated by addition of several different inorganic salts, such as sodium bromide (Wu *et al.* 2016) sodium azide (Huang *et al.*

2012; Kasimova *et al.* 2014), sodium thiocyanate (St Denis *et al.* 2013) and potassium iodide (Vecchio *et al.* 2015; Zhang *et al.* 2015; Freire *et al.* 2016; Huang *et al.* 2016, 2017, 2018a,c; Hamblin, 2017; Reynoso *et al.* 2017; Wen *et al.* 2017). The application of adjuvants can increase the photodynamic killing effect of diverse PS on different microorganisms, permitting the cationic PSs with few positive charges, neutral PSs, fullerenes and other dyes to successfully inactivate microorganisms with greater microbial inactivation rates in comparison to the use of the PS alone (Zhang *et al.* 2015; Vieira *et al.* 2018). This will probably decrease PS concentration and the treatment time and subsequently to diminish the total costs (Vieira *et al.* 2018).

Some studies demonstrated that the combination of KI with neutral porphyrins, fullerenes and other dyes gives rise to higher microbial inactivation rates when are compared to the use of the PSs alone (Zhang *et al.* 2015; Hamblin, 2017; Vieira *et al.* 2018). This salt is non-toxic and is already used in the antimicrobial, antiviral and antifungal therapy (Hamblin, 2017; Vieira *et al.* 2018). Zhang *et al.* (2015) studied the KI as potentiator of aPDT mediated by a C60 fullerene. The results showed that KI potentiated the ultraviolet A (UVA) or the white light-mediated killing of Gram-negative bacteria *Acinetobacter baumannii*, Gram-positive methicillin-resistant *Staphylococcus aureus* and fungal yeast *Candida albicans*, increasing the effect in 1–2 log (Zhang *et al.* 2015). This killing effect was also observed *in vitro* and *in vivo* using a mouse model with an infected skin abrasion (Zhang *et al.* 2015). Vecchio *et al.* (2015) evaluated the KI effect using Methylene Blue as PS in the photoinactivation of *E. coli* and *S. aureus*. This study showed that the addition of KI increased the bacterial killing in 4 and 2 log for *S. aureus* and *E. coli*, respectively, in a dose-dependent manner (Vecchio *et al.* 2015). The authors affirmed that the KI potentiator effect in these aPDT studies mediated by Methylene Blue was probably due to the formation of reactive iodine species that were quickly produced with a short lifetime (Vecchio *et al.* 2015). Since then, some other studies of the potentiation of aPDT effect using combinations of PSs and KI were reported. For example, methylene blue and new methylene blue were studied in the photoinactivation of oral *C. albicans* infection in a mouse model (Freire *et al.* 2016), BODIPY dyes in the photoinactivation of *E. coli*, *S. aureus* and *C. albicans* (Reynoso *et al.* 2017) and Photofrin in the photoinactivation of several Gram-negative bacteria (Huang *et al.* 2017). This approach was also efficient in aPDT of Gram-negative and Gram-positive bacteria mediated by Rose Bengal (Wen *et al.* 2017) and fullerenes (Huang *et al.* 2018a). In other study, Huang *et al.* (2018b) evaluated the KI effect using TPPS4 (anionic porphyrin and not able to bind to Gram-negative bacteria) and results demonstrated the anionic porphyrin in the presence of KI was able to photoinactivate *E. coli*. The combination of KI and methylene blue was also efficient to treat an urinary tract infection in a female rat model (Huang *et al.* 2018c). In other study, Vieira *et al.* (2018) studied the effect of KI as potentiator of aPDT mediated by a series of meso-tetraarylporphyrins positively charged at meso positions or at b-pyrrolic positions and the non-porphyrinic dyes Methylene Blue, Rose Bengal, Toluidine Blue O, Malachite Green and Crystal Violet in the photoinactivation of *E. coli*. The results indicate that KI has also the ability to potentiate the aPDT process mediated by some of the cationic PSs [Tri-Py(+)-Me, Tetra-Py(+)-Me, Form, Rose Bengal, Methylene Blue, Mono-Py(+)-Me, β-ImiPhTPP, β-ImiPyTPP, and

β-BrImiPyTPP] allowing a drastic reduction of the treatment time as well as of the PS concentration (Vieira *et al.* 2018). However, the efficacy of some porphyrinic and non-porphyrinic PSs [Di-Py(+)-Me opp, Di-Py(+)-Me *adj*, Tetra-Py, Toluidine Blue, Crystal Violet, and Malachite Green] was not potentiated by KI (Vieira *et al.* 2018) (Table 5). Recently, Vieira *et al.* 2019 evaluated the KI effect using cationic porphyrins (Form) in eradicating the Gram-positive bacteria (*S. aureus*), Gram-negative bacteria (*E. coli)*, fungal yeast (*C. albicans*) and T4-like bacteriophage as a mammalian virus model. The results showed that the presence of KI enhanced the photodynamic effect of this PS for all microorganisms studied, allowing the reduction of PS concentration and treatment time (Vieira *et al.* 2019). The combination Form/KI was also highly efficient in the elimination of biofilms of *E. coli*, *S. aureus* and *C. albicans* (Vieira *et al.* 2019). All these studied helped to elucidate the mechanism of action of KI potentiation. It was proposed, that the additional killing effect is instigated by many parallel reactions initiated by the reaction of $^1O_2$ with KI creating peroxyiodide (Figure 2), that can undergo to further decay by two different pathways, which are dependent on the degree of binding of the PS to the microbial cells (Vecchio *et al.* 2015; Zhang *et al.* 2015; Freire *et al.* 2016; Gsponer *et al.* 2016; Reynoso *et al.* 2016; Hamblin, 2017; Huang *et al.* 2017, 2018a; Kashef *et al.* 2017; Wen *et al.* 2017; Vieira *et al.* 2018; Vieira *et al.* 2019). One of the pathways (Figure 2) implies the formation of free iodine (I_2/I_3^-) and hydrogen peroxide (H_2O_2) (Vecchio *et al.* 2015; Zhang *et al.* 2015; Vieira *et al.* 2018). This compound can destroy microbial cells when produced in solution (Vecchio *et al.* 2015; Zhang *et al.* 2015; Vieira *et al.* 2018). However, it needs to achieve a satisfactory threshold concentration in order to develop a microbicidal effect (Vecchio *et al.* 2015; Zhang *et al.* 2015; Vieira *et al.* 2018). The quantity of free iodine formed depends on the amount of $^1O_2$ created, as well on the concentration of iodide anion present in the solution (Vecchio *et al.* 2015; Zhang *et al.* 2015; Vieira *et al.* 2018). The second pathway (Figure 2) presuppose a homolytic cleavage process, producing reactive iodine radicals ($I_2^{\cdot -}$) which are more toxic if generated very close to the target cells, once these radicals have short diffusion distance (Vecchio *et al.* 2015; Zhang *et al.* 2015; Vieira *et al.* 2018).

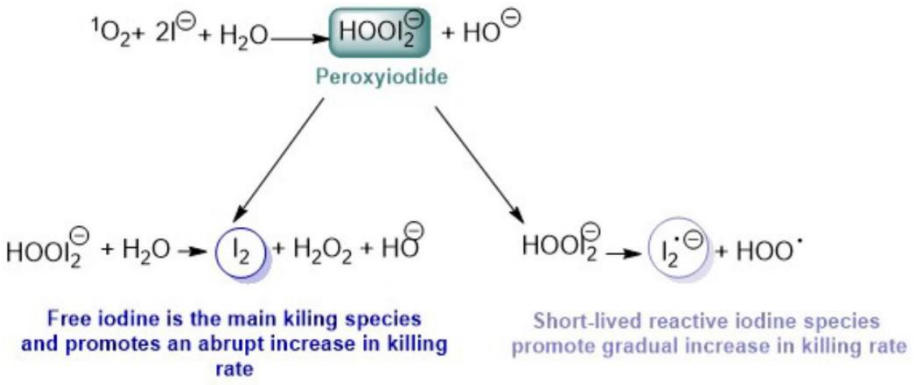

Figure 2. Schematic representation of the decomposition of peroxyiodide produced by the reaction of $^1O_2$ and KI (Vieira *et al.* 2018).

This killing function of the two species, I_2 and $I_2^{\cdot -}$, can be observed by the killing microbial profile, which can be verified by the decreasing of CFU mL^{-1} or PFU mL^{-1} throughout the irradiation time (Huang *et al.* 2018; Vieira *et al.* 2018). When free iodine (I_2) is produced, the inactivation effect is abrupt (Huang *et al.* 2018; Vieira *et al.* 2018). Whereas, a gradual inactivation effect is observed when the short-lived reactive iodine species ($I_2^{\cdot -}$) are the principal killing species (Huang *et al.* 2018; Vieira *et al.* 2018).

Table 5. Results obtained in the photoinactivation of bioluminescent *E. coli* using the combination of tested PSs at 5.0 µM and KI (Vieira *et al.* 2018).

	Mono-Py(+)-Me	Di-Py(+)-Me *opp*	Di-Py(+)-Me *adj*	Tri-Py(+)-Me	Tetra-Py(+)-Me	FORM	Tetra-Py	β-ImiPhTPP	β-ImiPyTPP	β-BrImiPhTPP	RB	MB	TBO	CV	MG
KI potentiate aPDT?	✓	✗	✗	✓	✓	✓	✗	✓	✓	✓	✓	✓	✗	✗	✗
KI causes a sharp decrease in the *E. coli* survival?	✗	-	-	✓	✓	✓	-	✗	✗	✗	✓	✓	-	-	-

✗ : No ; ✓ : Yes ;

1.5.8) POTENTIATION OF APDT BY HYDROGEN PEROXIDE (H_2O_2)

Hydrogen peroxide is an oxidizing agent used extensively in cleaning wounds and removing dead tissue (Feuerstein *et al.* 2006; Hamblin, *et al.* 2013). Some studies have indicated that the use of H_2O_2 associated with PDT gives increased killing of microorganisms (McCullagh and Robertson, 2006; Garcez *et al.* 2011; Hamblin, *et al.* 2013). In 2006, McCullagh and Robertson first reported a possible improvement of photoinactivation of the cyanobacterium *Synechococcus leopoliensis* using Methylene Blue in the presence of H_2O_2 (McCullagh and Robertson, 2006). In the same year, these authors showed the photoinactivation of *Chlorella vulgaris* by methylene blue and nuclear fast red combined with H_2O_2 under visible light irradiation (McCullagh and Robertson, 2006). Garcez *et al.* (2011) had similar results working with Methylene Blue in the presence of H_2O_2. These authors studied the antimicrobial photodynamic effect of Methylene Blue (60 µM) in the presence of H_2O_2 (10 mM, 100 mM and 1 M) in order to kill *S. aureus*, *E. coli* and *C. albicans* (Garcez *et al.* 2011). When H_2O_2 was added to Methylene Blue, there was an increased antimicrobial effect of around 70% *C. albicans* and *S. aureus*, and approximately of 60% for *E. coli*, for dose-dependent way (Garcez *et al.* 2011). According to Garcez *et al.* (2011), this effect could be due to a change in the type of ROS generated or increased microbial uptake of methylene blue. In other study, Hamblin (2013) affirmed that the H_2O_2 can modify the

membrane/envelope/capsid permeability and hence the probability of cellular accumulation of the PS, or it may be due to the membrane/envelope/capsid disruption caused by photoreaction which enable the penetration of H_2O_2 into the microorganism (Hamblin, *et al.* 2013).

1.6 WATER DISINFECTION BY APDT

The PDI of microorganisms in the context of the water disinfection and sterilization goes back to the 1970s (Bezman *et al.* 1978). However, the use of porphyrin derivatives as PS for the aim of treating water through the photodynamic process was only mentioned in scientific literature in the year 2000 (Almeida *et al.* 2009; Magaraggia *et al.* 2011). The practical application of photodynamic treatment to disinfect microbiologically polluted waters depends on various factors (Almeida *et al.* 2009; Magaraggia *et al.* 2011):

- Tthe removal of the PS after photodynamic treatment to avoid the release of PS to the environment;
- The use of photo-stable PS, that is PS which do not degrade under irradiation;
- The impact of this method on the structure of the natural non-pathogenic microbial communities;
- The toxicity of the PS to aquatic organisms at doses which induce marked mortality on microbial pathogens;
- The effect of chemical and physical properties of environmental waters;
- The possibility of using sunlight as light source.

The potential reuse of treated wastewater in agriculture (crop irrigation) was suggested in the research by Alouini and Jemli. (2001) and Jemli *et al.* (2002). Alouini and Jemli. (2001) showed the efficient PDI of helminth eggs by the tetracationic 5,10,15,20-tetrakis(1- methylpyridinium-4-yl)porphyrin (Tetra-Py(+)-Me) under visible light illumination, in clear water and in secondary treated wastewater. In other study, Jemli *et al.* (2002) evaluated the inactivation of faecal coliforms using a combination of a PS (Rose Bengal, Methylene Blue, cationic porphyrin) with sunlight. The results showed that the meso-substituted cationic porphyrin is more efficient and more photostable than methylene blue and rose bengal in wastewater (Jemli *et al.* 2002). The authors also tested the application of Tetra-Py(+)-Me at different concentrations (1.0, 5.0 and 10 μM) with sunlight irradiation (1235 mW cm^{-2}) during 240 min in order to inactivate faecal coliforms on wastewater samples after the secondary treatment (Jemli *et al.* 2002). At 5.0 μM and 10 μM, the faecal bacterial counts decreased about 2.9 and 2.4 log, respectively, after 60 min of irradiation (Jemli *et al.* 2002). After 240 min, a total cell survival reduction (> 4.0 log units) was achieved with both concentrations (Jemli *et al.* 2002). By increasing the duration of irradiation, the inactivation in bacteria increase, that compensate for a low concentration of sensitizer or for a less efficient type of sensitizer (Jemli *et al.* 2002). The authors considered the 5.0 μM concentration more

suitable to reduce faecal coliforms in wastewater since it allows obtaining a good treatment yield and it is more economic (Jemli *et al.* 2002). According to Alouini and Jemli (2001), the suspended solids (turbidity) were the most influential solution parameter on the efficiency of the photochemical process. Turbidity reduces light penetration, which reduces the PS excitation and the absorption (Alouini and Jemli, 2001). In fact, the decrease in log counts of faecal coliforms was ≈ 1.0 after 1 h of phototreatment by 5 μM Tetra-Py(+)-Me when suspended solids reached 50 mg L^{-1} (Jemli *et al.* 2002).

In a pioneering work, Bonnett et al. (2006) used PS incorporated into a polymeric membrane to disinfect water. The PSs were incorporated into translucent chitosan membranes by adsorption [5,10,15,20-tetrakis(p-hydroxyphenyl)porphyrin, p-THPP], by dissolution, casting [5,10,15,20-tetrakis(p-aminophenyl)porphyrin, p-TAPP], and by covalent attachment by reactive dyeing [zinc(II) phthalocyanine tetrasulfonic acid, ZnPcS] (Bonnett *et al.* 2006). The chitosan membrane containing the 5,10,15,20-tetrakis(4-aminophenyl)porphyrin (p-TAPP) caused a reduction to 1300 CFU mL^{-1} after 30 min of irradiation with the white light (Bonnett *et al.* 2006). However, the membrane prepared with ZnPcS4, was more effective and able to completely inactivate the *E. coli* after 30 min of irradiation (Bonnett *et al.* 2006). When the membrane prepared with ZnPcS4 was stored in the dark for nine months, the photodynamic action was still detectable demonstrating its thermodynamic stability (Bonnet *et al.* 2006). Villén *et al.* (2006) and Manjón *et al.* (2009) proposed immobilizing PSs from a polyazaheterocyclic Ru(II) group onto porous silicone in order to apply them for water disinfection. Recently, Valkov *et al.* (2019) immobilized the PSs Rose Bengal sodium salt, Rose Bengal lactone, methylene blue, and hematoporphyrin in polyethylene or polypropylene using a “green” method of co-extrusion, without addition of any chemicals, yielding polymeric strips and beads containing the PSs. The antibacterial efficiency of these immobilized PSs was tested against *S. aureus* and *E. coli* in batch and continuous regimes upon illumination with a white luminescent lamp (Valkov *et al.* 2019). All studied PSs demonstrated good efficacy in bacterial eradication (Valkov *et al.* 2019).

In last decade, our research group has developed a broad-spectrum of PS, namely cationic porphyrins, which can efficiently inactivate microorganisms in wastewater (Carvalho *et al.* 2007). Two of the cationic porphyrins were used to photoinactivate faecal coliforms and faecal enterococci in wastewater samples from a secondary-treated sewage plant (Carvalho *et al.* 2007). The results showed that the two cationic porphyrins inactivated 94 – 99.8% of the faecal coliforms at 5.0 μM upon white light at low light fluence (9 mW cm^{-2}) after 270 min of irradiation (Carvalho *et al.* 2007).

Later, seven synthetic cationic meso-substituted porphyrins with one to four charges were tested to photoinactivate *Enterococcus faecalis* and *E. coli* (7 log CFU mL^{-1}) with low light irradiance (4 mW cm^{-2}) (Carvalho *et al.* 2010). The results showed that the tri-(Tri-Py(+)-Me-PF and Tri-Py(+)-Me-CO_2Me) and the tetra-cationic PS (Tetra-Py(+)-Me) at 5.0 μM were the most efficient ones (Carvalho *et al.* 2010). The complete photoinactivation of *E. coli* (7 log CFU mL^{-1}) was observed after 90 min with Tri-Py(+)-Me-PF and Tri-Py(+)-Me-CO_2Me and after 270 min with Tetra-Py(+)-Me (Carvalho *et al.* 2010). These

results suggested that PDI of faecal bacteria can be a possibility for wastewater disinfection under natural light conditions (Carvalho *et al.* 2010).

Alves *et al.* (2008) reported the photoinactivation of a recombinant bio-luminescent *E. coli* strain whose light emission decreased more than 4 log with the three porphyrins used (Tetra-Py(+)-Me, Tri-Py(+)-Me-PF and Tri-Py(+)-Me-CO_2Me), but, Tri-Py(+)-Me-PF (5.0 μM) was the most efficient compound. These results were observed both with artificial white light (4 mW cm^{-2}, 64.8 J cm^{-2}) and with sunlight (~62 mW cm^{-2}, 1004.4 J cm^{-2}) after 90 – 270 min (Alves *et al.* 2008). In same year, six porphyrins derivates (Tetra-Py(+)-Me, Tri-Py(+)-Me-PF, Tri-Py(+)-Me-CO_2Me, Tri-Py(+)-Me-CO_2H, Di-Py(+)-Me-Di-CO_2-adj and Di-Py(+)-Me-Di-CO_2H-opp) were tested on bacteriophages isolated from wastewater, using white light (40 W m^{-2}), and 5.0 μM of porphyrin (Costa *et al.* 2008). The tetra- and tricationic porphyrins inactivated the bacteriophage T4-like to the limits of detection (reduction of ~7 log), but dicationic porphyrins did not lead to a significant decrease in concentration of the bacteriophage (Costa *et al.* 2008). The authors concluded that the tetra- and tricationic porphyrins can be used as a new method for inactivating sewage bacteriophages that are frequently used as human enteric virus indicators (Costa *et al.* 2008). The complete inactivation of viruses with low light intensity means that this methodology can be used even on cloudy days and during winter, opening the possibility to develop new technologies for wastewater treatment (Costa *et al.* 2008). Later, in order to establish the best conditions for an efficient photoinactivation of somatic bacteriophages, these authors evaluated how light source, light dose and fluence rate, in the presence of efficient PS, can affect the viral photoinactivation of a T4-like sewage bacteriophage (Costa *et al.* 2010). The research was carried out using white PAR light delivered by fluorescent PAR lamps (40 mW cm^{-2}), sun light (600 mW cm^{-2}) and an halogen lamp (40–1690 mW cm^{-2}) and two cationic PSs (Tetra-Py(+)-Me, Tri-Py(+)-Me-PF) at 0.5, 1.0 and 5.0 μM. The results showed that the efficacy of the bacteriophage photoinactivation is correlated not only with the PS and its concentration but also with the light source, energy dose and fluence rate applied (Costa *et al.* 2010). Both PSs at 5.0 μM were able to inactivate the bacteriophage T4-like to the limit of detection (reduction of ~7 log $PFUmL^{-1}$) for each light source and fluence rate. However, depending of the light parameters, different irradiation times are required (Costa *et al.* 2010).

Although there is need for scientific knowledge on disinfection of hospital wastewater, there is only one report, on the use of photodynamic treatment on clinical MDR bacteria in hospital wastewaters (Almeida *et al.* 2014). In this study was evaluated the efficiency of photoinactivation on four multi-drug resistant strains of *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *A. baumannii* in buffered solution and in hospital wastewater, using 5.0 μM of Tetra-Py^{+}-Me and white light (64.8 J cm^{-2}) (Almeida *et al.* 2014). The results showed an efficient inactivation of multidrug-resistant bacteria in buffered solution (reduction of 6 – 8 log CFU mL^{-1}). In wastewater, the photoinactivation of the four bacteria was also effective and the decrease in bacterial concentration occurred even sooner (Almeida *et al.* 2014). The authors assigned this difference to dissolved compounds in the hospital wastewater, such as antibiotics (Almeida *et al.* 2014).

More recently Bartolomeu *et al.* (2017), suggested that the PDI can be applied to wastewater treatment to inactivate microorganisms but also to photodegrade chemicals. In this study was evaluated the efficiency of photoinactivation on *E. coli in* buffered solution and in filtered wastewater, using 10 µM of Tetra-Py(+)-Me and artificial white light (40 mW cm^{-2}) (Bartolomeu *et al.* 2017). The potential of PDI to inactivate the native bacteria present in wastewater was evaluated in non-filtered wastewater. It was also tested if the same PDI protocol was able to induce phototransformation of phenol. In both conditions (PBS and filtered wastewater), aPDT was an efficient antimicrobial method (Bartolomeu *et al.* 2017). These results show that in lower concentrations of microorganisms in phosphate-buffered saline (PBS), the aPDT is more efficient, yet, the inactivation rate was higher at lower periods of treatment in the assays in the filtered wastewater (Bartolomeu *et al.* 2017). In non-filtered wastewater, the cationic porphyrin was effective against both bacterial groups tested (Bartolomeu *et al.* 2017). The phenol is the most abundant contaminant in industrial wastewater (Kujawski *et al.* 2004; Mahvi *et al.* 2007). The contamination of natural waters with phenol is a problem in terms of environmental considerations owing to its high toxicity (Kujawski *et al.* 2004; Mahvi *et al.* 2007). In this study, phenol was photo-degraded (20 mg mL^{-1}) after 60 min of irradiation with solar light at an irradiance interval between 389 and 1206 mW cm^{-2} and a Tetra-Py(+)-Me concentration of 25 µM (Kujawski *et al.* 2004; Mahvi *et al.* 2007).

CHAPTER 2 - PHOTODYNAMIC INACTIVATION OF T4-LIKE BACTERIOPHAGE IN WASTEWATER

Photodynamic Inactivation of T4-like Bacteriophage in Wastewater

Abstract:

Pathogenic viruses are frequently present in marine and estuarine waters due to antimicrobial ineffective treatment performed to wastewater (WW) in effluents plants, which, consequently, affect water quality and human health. Chlorination, one of the most common methods used to ensure microbiological safety in tertiarily treated effluents, may lead to the formation of toxic chemical disinfection by-products through the reaction with organic matter present in the effluents. aPDT can be a promising approach for the inactivation of pathogens without the formation of toxic known by-products. Additionally, pevious studies have reported the potentiator effect on aPDT in combination with some compounds as KI and H_2O_2. In the present study, it was evaluated the aPDT efficiency of a PS based on a low-cost formulation constituted by five cationic porphyrins (Form) and the potentiation effect by KI and H_2O_2 in the inactivation of a T4-like bacteriophage in different aqueous matrices with different organic matter content and several Form concentrations. The results showed that the efficiency of bacteriophage photoinactivation is correlated with the concentration of the used PS and the increasing of the organic matter promotes a decreasing in the aPDT efficiency. Form can be an effective alternative to control viruses in WW, particularly if combined with H_2O_2. However, the combination of aPDT with KI, did not potentiate the bacteriophage inactivation.

Keywords: Antimicrobial photodynamic therapy, porphyrin Form, wastewater, bacteriophage T4-like, potassium iodide, hydrogen peroxide

1. INTRODUCTION

Wastewater containing pathogens is subject of concern, affecting the quality of the receiving waters where they are discharged. The disposal of inadequately treated wastewater is the main source of microorganisms in the aquatic environment (Sedmak *et al.* 2005; Albinana-Gimenez *et al.* 2006; Okoh *et al.* 2010). Even though wastewater is secondarily treated before launched into seawater and rivers, this effluent contains high concentrations of microorganisms, but dilution makes it acceptable in terms of quality indicators. However, emerging of MDR microorganisms brought serious risks when wastewater is not properly treated, contributing to a widespread of emerging pathogenic strains.

Wastewater contains high concentrations of pathogenic microorganisms, including bacteria, viruses, fungi and parasites (Dumontet *et al.* 2001; Ramírez-Castillo *et al.* 2015; Al-Gheethi *et al.* 2018), and viruses are among the most persistent pathogens (Atabakhsh *et al.* 2019). Despite the advances in WWTP, a large number of human enteric viruses are discharged into the aquatic environment (Atabakhsh *et al.* 2019), including estuarine and marine environments, through offshore sewage outfalls,

sewage treatment plants and septic tanks. Human enteric viruses are considered emerging waterborne pathogens, (Atabakhsh *et al.* 2019) representing a problem for public health, economy and environmental ecology (Lee and Kim 2002; Hamza *et al.* 2009; Rodríguez-Díaz *et al.* 2009). Enteric viruses such as enterovirus, norovirus, rotavirus, adenovirus, astrovirus and hepatovirus are responsible for the spread of some worrying diseases like gastroenteritis, poliomyelitis (Tesini, 2019), myocarditis (Flynn *et al.* 2017), encephalitis, hepatitis and are frequently found in aquatic environments (Bosch *et al.* 2008; Rodríguez-Díaz *et al.* 2009).

In order to reduce the concentration of pathogens in wastewater to levels comparable to those found in natural waters, tertiary treatment, usually using chlorine, ozone or ultraviolet light, is necessary (to achieve the guidelines of the World Health Organization (WHO) and the United States Environmental Protection Agency (EPA) standards for microorganisms presence in water) (Al-Gheethi *et al.* 2018). However, these treatments are expensive, toxic to aquatic species, and induce genetic damages to microorganisms (Costa *et al.* 2012). Among the tertiary treatments, chlorination was the first chemical water disinfection approach to be implemented as a standard process (Gray, 2014) and currently, it is the most common method of ensuring microbiological safety in tertiary effluents since it effectively inactivates bacteria and viruses (Costa *et al.* 2008; Al-Gheethi *et al.* 2018). Though, its massive utilization may lead to the formation of disinfection by-products with potential health hazards, as carcinogenic chlorinated disinfection by-products when reacting with organic compounds present in the wastewater (Costa *et al.* 2008; Gray, 2014; Al-Gheethi *et al.* 2018). So, for the reduction of waterborne dissemination diseases, new and safe treatments should be developed (Jemli *et al.* 2002; Carvalho *et al.* 2007; Bartolomeu *et al.* 2017).

aPDT can be a very promising alternative to those treatments. aPDT involves the use of a PS which in the presence of visible light and molecular oxygen produces reactive oxygen species (ROS), such as free oxygen radicals and singlet oxygen ($^1O_2$). These reactive species are responsible for the oxidation of several cellular components conducting rapid cell inactivation. aPDT has shown to be a powerful method for viruses inactivation, with several studies showing its effectiveness, either against adenovirus (Schagen *et al.* 1999), herpes simplex virus (HSV) (Müller-Breitkreutz *et al.* 1995; Smetana *et al.* 1998), human immunodeficiency viruses (HIV) (Lenard *et al.* 1993; North *et al.* 1994; Rywkin *et al.* 1994; Müller-Breitkreutz and Mohr, 1998; Vzorov *et al.* 2002), vesicular stomatitis virus (VSV) (Lenard *et al.* 1993; Abe and Wagner, 1995; Käsermann and Kempf, 1997; Moor *et al.* 1997; Wagner *et al.* 1998; Lim *et al.* 2002), bovine viral diarrhoea virus (BVDV) (Sagristá *et al.* 2009), encephalomyocarditis virus (EMCV) (Sagristá *et al.* 2009), hepatitis A (HAV) (Casteel *et al.* 2004) and hepatitis C virus (HCV) (Müller-Breitkreutz and Mohr, 1998), as well as influenza virus (Lenard *et al.* 1993) and enterovirus 71 (Wong *et al.* 2010). The effect of aPDT on mammalian viruses has also been studied using bacterial viruses (bacteriophages) as surrogates, due to a variety of reasons, including the one that they are as resistant as the mammalian viruses to the water treatment and environmental factors (Leclerc *et al.* 2000), with very positive results of efficient photoinactivation (Abe and Wagner, 1995; Schneider *et al.*

1998; Gábor *et al.* 2001; Egyeki *et al.* 2003; Casteel *et al.* 2004; Badireddy *et al.* 2007; Zupán *et al.* 2008; Costa *et al.* 2008, 2010, 2011; Vieira *et al.* 2019).

A T4-like bacteriophage, a Caudovirales order member with an elongated icosahedral head and a contractile tail (Myoviridae family) (Pereira *et al.* 2017) was used in the present study, as a model of enteric viruses (Taj *et al.* 2014). Bacteriophages are frequently used as indicators of the presence of human enteric pathogens and microbial faecal pollution which may lead to consequent public health risks. Several studies have shown a successful photoinactivation of bacteriophages (Kadish *et al.* 1967; Costa *et al.* 2008; Costa *et al.* 2010; Costa *et al.* 2011) depending, their effectiveness, on variables as the structural composition of the PS, including the number and position of positive charges and hydrophobicity (Costa *et al.* 2008).

Recently, a PS formulation (Form), based on a non-separated mixture of five cationic *meso*-tetraarylporphyrins, has proven its high efficiency in the photoinactivation of microorganisms such as *S. aureus* (a Gram-positive bacteria), *E. coli* (a Gram-negative bacteria), *Pseudomonas syringae* pv. actinidiae (a Gram-negative bacteria), as well as *C.albicans* (a fungi) (Marciel *et al.* 2018; Martins *et al.* 2018; Vieira *et al.* 2018), being so considered a relevant alternative to highly efficient purified PSs, such as Tri-Py(+)-Me, since its production costs, as well as production time, is significantly reduced when compared to the purified cationic porphyrin (Vieira *et al.* 2018). This PS formulation is composed of the combined PSs Mono-Py(+)-Me (19%), Di-Py(+)-Me *opp* and Di-Py(+)-Me *adj* (20%), Tri-Py(+)-Me (44%) and Tetra-Py(+)-Me (17%).

The use of PS combined with some inorganic salts such as sodium thiocyanate (St. Denis *et al.* 2013), sodium bromide (Wu *et al.* 2016), sodium azide (Huang *et al.* 2012; Kasimova *et al.* 2014), potassium iodide (Vecchio *et al.* 2015; Zhang *et al.* 2015; Huang *et al.* 2016, 2017, 2018a, b, c; Hamblin, 2017; Reynoso *et al.* 2017; Wen *et al.* 2017) demonstrated to improve of aPDT efficiency. Several studies *in vitro* and *in vivo* have shown that the addition of KI can potentiate the aPDT effect on bacteria (such as *A. baumannii, P. aeruginosa)* and fungi (*C. albicans*) and can reduce the incidence of regrowth after treatment due to the production of free iodine/triiodide (I_2/I_3^-), iodine radicals ($I_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), longer-lived reactive species than $^1O_2$ that may remain active even after the aPDT treatment (Zhang *et al.* 2015; Vecchio *et al.* 2015; Gsponer *et al.* 2016; Huang *et al.* 2016, 2017, 2018a, b, c; Hamblin 2017; Kashef *et al.* 2017; Reynoso *et al.* 2017; Wen *et al.* 2017; Vieira *et al.* 2018). Vieira *et al.* (2018) reported that the combination of Form with KI was highly efficient in the photoinactivation of *E. coli* (6 log after 30 min of irradiation) when compared to the photoinactivation of this bacterium in the presence of Form alone (4 log after 60 min of irradiation).

According to some authors, hydrogen peroxide can also be combined effectively to PS as an enhancer of the aPDT effectiveness (McCullagh and Robertson, 2006; Hamblin, *et al.* 2013). The radical species produced from the PS would react with H_2O_2 producing hydroxyl radicals, thereby increasing the number of radical species available in solution to damage viral structures as proteins, lipids and nucleic acids (Costa *et al.* 2014; Almeida *et al.* 2015; Alves *et al.* 2015).

To evaluate the photodynamic effect of Form and its potentiation effect by KI and H_2O_2 in the inactivation of bacteriophages in wastewater, experiments were carried out in PBS, used as a standard aqueous matrix, and in filtered and non-filtered wastewater loaded with a bacteriophage T4-like.

2. MATERIALS AND METHODS

The effectiveness of aPDT against the bacteriophage was evaluated using three different types of microcosms: (i) PBS; (ii) filtered wastewater; (iii) non-filtered wastewater. The first microcosm (PBS) was used as the standard condition. Buffered solutions, such as PBS, are useful to evaluate the behavior and efficacy of the PSs in a medium without organic matter and cell interference to select the best aPDT conditions. However, as the composition of the test matrix is an influencing factor for aPDT efficiency, to pave the realistic application, it is required to test the aPDT protocol in a relevant setting, such as is the case of this study, in filtered and non-filtered wastewater.

In filtered wastewater, assays were carried with different pore-sized membranes (0.22, 0.30 and 0.45 µm) to evaluate the effect of the dissolved organic matter in the efficiency of aPDT protocol. Also, a wide range of Form concentrations (from 1.0 to 10 µM) with and without the addition of KI were tested in filtered wastewater with 0.45 µm pore-sized membrane, to maintain most of the organic content (minimizing the dissolved organic matter suppression) and at the same time to allow the removal of most of the microorganisms naturally present in wastewater. Then, the possible extended effect of longer-lived reactive species such as I_2/I_3^-, $I_2^{\cdot-}$ and H_2O_2 was evaluated during dark incubation after aPDT protocol.

Lastly, the potentiator effect of H_2O_2 added to Form was tested in non-filtered wastewater. The volumes of H_2O_2 were added to the final suspension, in order to reach concentrations of 2, 5 and 9% in a final volume of 1mL of suspension.

2.1 WW SAMPLES

Secondarily treated wastewater composite samples were collected at a WWTP located at the littoral center of Portugal. This facility serves a wide geographic area, which encompasses several industrial as well as urban areas served by a sanitary network. Composite samples were representative of a period of 24 h and were collected on different days, encompassing a period of nine months in total (from October until June). Samples were collected in the early morning, protected from light and refrigerated at 4 ºC. Depending on the purpose of the assays, some of the collected samples were filtered using sterile 0.22, 0.30 and 0.45 µm pore-size membranes (Millipore Bedford, MA, USA), to eliminate residual bacteria and organic material.

2.2 BACTERIAL STRAINS AND GROWTH CONDITIONS

E. coli (ATCC 13706) was used in this study as the *E. coli* bacteriophage T4-like host. Fresh bacterial culture was maintained in Tryptic Soy Agar (TSA, Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 30 mL of Tryptic Soy Broth (TSB, Liofilchem, Italy) and grown overnight at 37 ºC under stirring (120 rpm). An aliquot (300 µL) of the previously mentioned culture was transferred into 30 mL of fresh TSB under the same prior growth conditions to reach the stationary phase of approximately 10^8 colony-forming units per mL (CFU mL^{-1}).

2.3 BACTERIOPHAGE PREPARATION

A T4-like bacteriophage (bacteriophage phT4A) previously isolated from a sewage network of Aveiro, using *E. coli* as the host (Costa *et al.* 2008). The phage suspensions were prepared from the bacteriophage stock previously prepared in SM buffer [0.1 M NaCl (Merck KGaA, Darmstadt, German), 8 mM $MgSO_4$ (Merck KGaA), 20 mM Tris-HCl (Merck KGaA), 2% (w/v) gelatin, pH 7.5]. Three hundred microliters of the bacteriophage stock were added to 30 mL of *E. coli* in the exponential growth phase. The suspension was grown overnight and incubated at 25 ºC at 50 rpm. The lysates were incubated with chloroform, for the elimination of bacterial content, (final volume of 1%) for 1 h at 120 rpm. After incubation, the lysate was centrifuged at 13 000 rpm for 10 min at 4 ºC, to remove intact bacteria or bacterial debris. Bacteriophage suspension was stored at 4 ºC and the titer was determined by the double-layer agar method (Adams, 1950). Successive dilutions of the bacteriophage suspension were performed in PBS solution [137 mM NaCl (Merck KGaA), 2.7 mM KCl (Merck KGaA), 8.1 mM $Na_2HPO_4.2H_2O$, 1.76 mM KH_2PO_4 (Merck KGaA), pH 7.4], and 500 µL of each dilution, together with 200 µL of fresh bacterial culture, and were mixed with 5 mL of TSB 0.6% top agar layer (30 g/L TSB (Liofilchem), 6 g/L agar (Liofilchem), 0.05 g/L $CaCl_2$ (Merck KGaA), 0.12 g/L $MgSO_4$ (Merck KGaA), pH 7.4) and placed over a TSA plate. The plates were incubated at 37 ºC for 18–24 h. After incubation, the number of plaques was counted, and the results expressed as plaque-forming units per milliliter (PFU mL^{-1}).

2.4 aPDT PROCEDURE

2.4.1 PHOTOSENSITIZER

Stock solution of Form was prepared at 500 µM in dimethyl sulfoxide (DMSO) and kept in the dark. Form is a non-separated mixture of five *meso*-tetraarylporphyrins, composed by 5-(1-methylpyridinium-4-yl)-10,15,20-tris(pentafluorophenyl)-porphyrin mono-iodide [Mono-Py(+)-Me], 5,15-bis(1-methylpyridinium-4-yl)-10,20-bis(pentafluorophenyl)porphyrin di-iodide [Di-Py(+)-Me *opp*] 5,10-bis(1-methylpyridinium-4-yl)-15,20-bis(pentafluorophenyl)-porphyrin di-iodide [Di-Py(+)-Me *adj*], 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide [Tri-Py(+)-Me] and

5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide [Tetra-Py(+)-Me] and was synthetized according with the literature (Simões *et al.* 2016; Marciel *et al.* 2018). Before each assay, the stock of Form solution was sonicated for 30 min at room temperature (ultrasonic bath, Nahita 0.6 L, 40 kHz).

2.4.2 KI AND H_2O_2 SOLUTIONS PREPARATION

Potassium iodide (KI) (Merck KGaA) solutions were prepared at 5.0 M in sterile distilled water immediately before each experiment.

Hydrogen peroxide (H_2O_2) (Merck KGaA) solutions were prepared adjusting the concentration of the stock solution at 30% to the final concentrations of 2, 5 and 9% used in the experiments.

2.4.3 IRRADIATION CONDITIONS

The aPDT assays were carried out under artificial white light conditions. LED projectors (20 W of power, ~230V of voltage and with a frequency of ~50Hz) (EL®MARK) were used. In previous studies performed by Vieira *et al* (2019) in PBS, it was applied a light irradiance of 25 mW cm^{-2}, however, since these studies will be applied in wastewater (complex matrix), light irradiance was adjusted to 50 mW cm^{-2} and measured with a laser power and energy meter (RoHS) FieldMaxII-TOP combined with a high-sensitivity thermopile sensor PS19Q (Coherent, California, United States).

2.4.4 APDT ASSAYS IN PBS

Viera *et al* (2019) realized similar studies in PBS with a maximum concentration of Form at 2.5 µM. Once the following assays will be later performed in wastewater, a far more complex matrix comparing with PBS, for this study, the efficiency of the Form was tested at higher and different concentrations, 5.0 and 10 µM. Same concentrations were applied in PBS in order to make further comparisons of efficiency between the different matrices. The efficiency was evaluated through quantification of the number of bacteriophages in PBS. Bacteriophage at a concentration of 10^8 PFU mL^{-1} was tenfold diluted in PBS and distributed in sterilized glass beakers.

The appropriate volume of Form was added to the samples to achieve a final concentration of 5.0 and 10 µM. In these experiments, two controls were simultaneously performed: light control (LC) and dark control (DC). LC included bacteriophage suspension and was subjected to the same light conditions as the samples; DC included bacteriophage suspension, and was subjected to the same concentrations as the samples, but protected from light during the assays, wrapped in aluminum foil. Samples and controls were remained in the dark under stirring for 10 min at room temperature, to promote the PS binding to the bacteriophage particles, before each assay. Then, samples and light

controls were exposed to light at 50 mW cm^{-2}, for a total of 270 min of irradiation. Aliquots of samples and controls were collected at intermediate times of light exposure, tenfold diluted in PBS and drop plated (5.0 µL), in duplicate, in Petri dishes previously prepared with TSA and a layer of TSA soft with the bacteriophage host, *E. coli*. The Petri dishes were incubated at 37 ºC for 12 h and the number of lysis plaques was counted. The results were expressed as PFU mL^{-1}. Three independent assays, with two replicates, for each condition, were performed.

2.4.5 aPDT Assays performed in Filtered Wastewater

To evaluate the influence of the organic matter present in wastewater in the aPDT efficiency, assays with wastewater filtered by three different pore-sized membranes (0.22, 0.30 and 0.45 µm) were carried out.

The aPDT assays in filtered wastewater by 0.45 µm pore-size membrane were done with different concentrations of Form (1.0, 2.0, 3.0, 4.0, 5.0 and 10 µM) and were tested with (Form+KI) and without (Form) the addition of KI at 100 mM. The aPDT assays in filtered wastewater by 0.22 and 0.30 µM were performed at just one Form concentration (10 µM).

The bacteriophage suspension (at a concentration of 10^8 PFU mL^{-1}) was tenfold diluted in 0.45 µm filtered wastewater and distributed in a sterile 96 well microplate. The appropriate volumes of Form or Form+KI were added to the samples to achieve a final concentration of Form at 1.0, 2.0, 3.0, 4.0, 5.0 and 10 µM and KI at 100 mM. Once again, dark and light controls were carried out during the aPDT assays (at the same conditions as described above). A pre-irradiation period of 10 min in the dark, at room temperature and stirring was carried out and, then, samples and light controls were exposed to artificial light with an irradiance of 50 mW cm^{-2}. Aliquots of the samples and controls were collected at predefined times of light exposure. The bacteriophage suspensions were serially diluted in PBS and plated with their hosts by the drop plated method. The Petri dishes were incubated at 37 ºC for 12 h and the number of lysis plaques was counted, and the obtained results were expressed as PFU mL^{-1}. Three independent assays, with two replicates, for each condition, were performed.

The assays in 0.45 µm filtered wastewater was also used to test if the longer-lived reactive species, free iodine/triiodide (I_2/I_3^-), iodine radicals ($I_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), generated when aPDT is done in the presence of KI, can reduce the incidence of regrowth after treatment. These aPDT assays were performed with Form at 5.0 µM and the addiction of KI at 100 mM, since previous studies were applied with the same concentrations (Vieira *et al.* 2018). In these assays, after 15 and 30 min of irradiation, the samples were incubated in the dark at room temperature. For the samples irradiated during 15 min, aliquots were collected immediately after the 15 min of irradiation (0 min) and after 15, 30, 45, 60 and 90 min of dark incubation. For the ones irradiated during 30 min, less time-spaced aliquots were taken, so immediately after the irradiation period (0 min) and after 5, 10, 15, 30, 45, 60 and 90 min of dark incubation.

Three independent assays, with two replicates, for each condition, were performed.

2.4.6 aPDT Assays performed in Non-filtered Wastewater

To evaluate if the efficiency of aPDT to inactivate bacteriophages was maintained in raw wastewater, assays with Form at 10 μM were performed. To evaluate if the aPDT efficiency was potentiated by H_2O_2, assays with Form (at 5.0 and 10 μM), with H_2O_2 at 2, 5 and 9% (Form+H_2O_2) were also performed. The assays preparation was as described above. Aliquots of the samples and controls were collected at intermediate times of light exposure and determined for bacteriophage concentration as described above.

Three independent assays, with two replicates, for each condition, were performed.

2.5 Statistical Analysis

The statistical analysis was performed on the data resultant of three independent assays done in duplicate for each condition tested. The statistical analysis was done with GraphPad Prism. Normal distributions were checked by the Kolmogorov–Smirnov test. A two-way ANOVA and Tukey's multiple comparisons test was applied to assess the significance of the differences between the bacteriophage concentration along with the aPDT treatments. A p value < 0.05 was considered to be statistically significant.

3. Results

3.1 aPDT Assays in PBS

The results of the aPDT assays in PBS with Form at 5.0 and 10 μM are presented in Figure 3. When Form was used at 5.0 μM, the content in bacteriophage decreased more than 7 log PFU mL^{-1} after 270 min of treatment (810 J cm^{-2} light dose), when compared to the sample before aPDT (p value < 0.0001), occurring the most abrupt decrease during the first 90 min of irradiation (270 J cm^{-2} light dose) with an inactivation of ca. 5 log PFU mL^{-1} (p value < 0.0001). When Form concentration was doubled (10 μM), the inactivation efficiency was greatly increased and an inactivation to the detection limit of the method of more than 7 log PFU mL^{-1} was reached just after 30 min (90 J cm^{-2} light dose) of aPDT, when comparing the bacteriophage content in the sample before the treatment (p value < 0.0001). The Form concentration affected significantly the bacteriophage inactivation. After 30 min of treatment (90 J cm^{-2} light dose), the bacteriophage inactivation with Form at 5.0 (decrease of 1.4 log PFU mL^{-1}) and 10 μM showed a sharp difference of ca. 6 log PFU mL^{-1} between the two concentration conditions tested (p value < 0.0001).

Both light (LC) and dark [DC (Form)] controls remained constant along the experiment period, meaning that neither white light radiation alone has no effect on the viral particles viability, nor Form without light irradiation has a toxic effect in the bacteriophage particles.

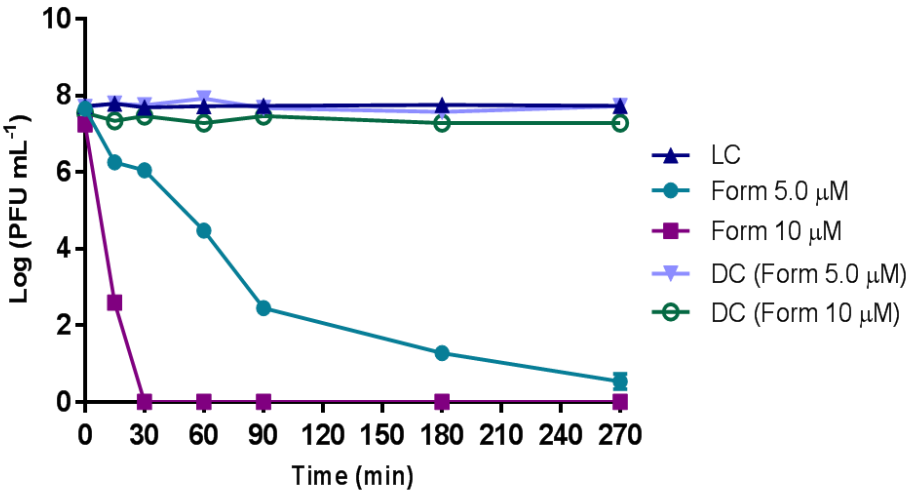

Figure 3. Inactivation of T4-like bacteriophage during aPDT, in PBS, with Form at 5.0 and 10 μM, for 270 min of irradiation with artificial white light (50 mW cm^{-2}). The values are expressed as the mean of three independent experiments; error bars represent the standard deviation (SD) between the experiments. In some cases, SD bars are covered behind the symbols.

3.2 aPDT Assays in Filtered Wastewater

The results obtained in the filtered wastewater are shown in Figure 4. In the assays with 0.20 and 0.30 μm filtered wastewater with Form at 10 μM it is shown that the efficiency of the bacteriophage inactivation was significantly increased (*p* value < 0.0001), inactivating the bacteriophage to the limit of detection of the method after 5 min (15 J cm^{-2} light dose). When the wastewater was filtered by 0.45 μm, a bacteriophage reduction to the detection limit was observed only after 15 min (45 J cm^{-2} light dose) of treatment (*p* value < 0.0001), showing that the amount of dissolved organic matter should play an important role in the aPDT efficiency (Figure 4).

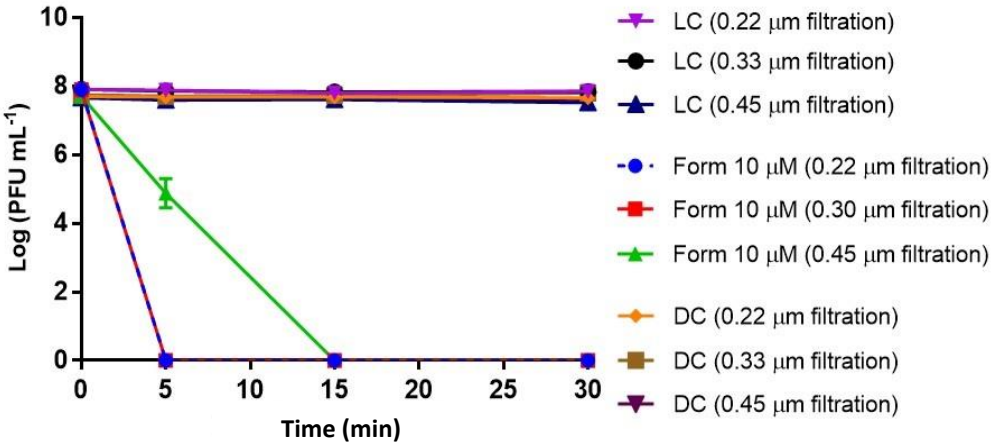

Figure 4. Inactivation of T4-like bacteriophage during aPDT, in filtered wastewater (0.22, 0.30 and 0.45 μm filtration) with Form at 10 μM, for 30 min of irradiation with white light (50 mW cm^{-2}). The values are expressed as the mean of three independent experiments; error bars represent the standard deviation (SD) between the experiments. In some cases, SD bars are covered behind the symbols.

The results obtained in the photoinactivation of bacteriophage with a wide range of Form concentrations (1.0, 2.0, 3.0, 4.0, 5.0 and 10 μM) on filtered wastewater, both in the presence and absence of KI (100 mM) are present in Figure 5. These assays were conducted in 0.45 μm filtered wastewater to minimize the dissolved organic matter suppression (maintaining most of the organic content) but allowing the removal of the majority of the suspended microorganisms in wastewater. In general, the results have shown that the use of KI does not potentiate the aPDT efficiency in filtered wastewater. Moreover, the retarding effect of KI on aPDT inactivation efficiency is either significant (*p* value < 0.0001) or does not promote any beneficial effect whatsoever.

The most significantly efficient inactivation occurred when Form was used at 10 μM without the addiction of KI (*p* value < 0.0001), after 15 min of irradiation (45 J cm^{-2} light dose). After 30 min of irradiation (90 J cm^{-2} light dose), the detection limit of the method was reached where Form was used at concentrations of 3.0, 4.0 and 5.0 μM, without KI. After 60 min of irradiation (180 J cm^{-2} light dose), inactivation to the detection limit of the method with Form at 2.0 μM without KI was observed. Also, when Form was used at 3.0 and 5.0 μM in combination with KI at 100 mM, inactivation to the detection limit was observed. When Form was used at 2.0 μM in combination with KI, the inactivation to the detection limit was reached only after 90 min of irradiation (270 J cm^{-2} light dose). For Form at 1.0 μM with and without the addition of KI, a reduction of 2.9 log and 0.85 log PFU mL^{-1} was observed after 180 min (540 J cm^{-2} light dose), but the detection limit of the method was not reached.

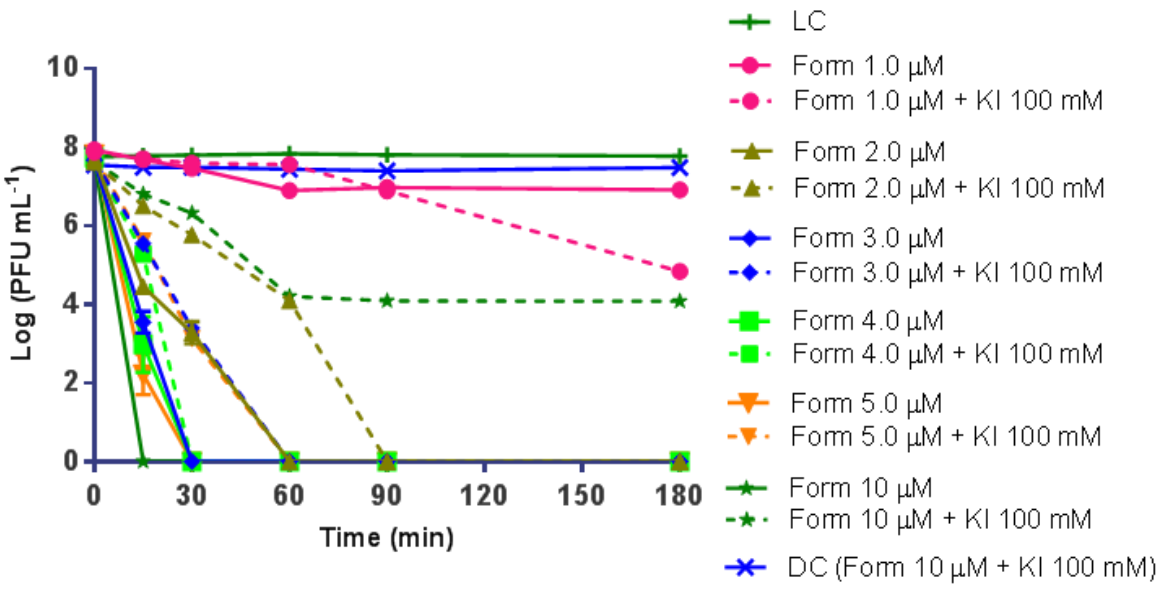

Figure 5. Inactivation of T4-like bacteriophage during aPDT, in filtered wastewater (0.45 µm filtration) with Form at different concentrations (at 1.0, 2.0, 3.0, 4.0, 5.0 and 10 µM) alone and in combination with KI (at 100 mM), for 180 min of irradiation with white light (50 mW cm^{-2}). The values are expressed as the mean of three independent experiments; error bars represent the standard deviation (SD) between the experiments – to note that in some cases SD bars are covered behind the symbols.

aPDT experiments with a PS as Form in combination with KI, were performed in a suspension with 0.45 µm filtered wastewater, which was irradiated for 15 or 30 min and, then, kept in the dark were done. The objective was to evaluate the possible effect of reactive species formed when KI reacts with $^1O_2$, namely free iodine/triiodide (I_2/I_3^-), iodine radicals ($I_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) with a longer half-time that ROS formed during aPDT treatments with Form. The results of 15 (Figure 6A) and 30 min (Figure 6B) of irradiation showed no significant effect along the dark incubation period (*p* value > 0.05) for the Form alone and the Form combined with KI.

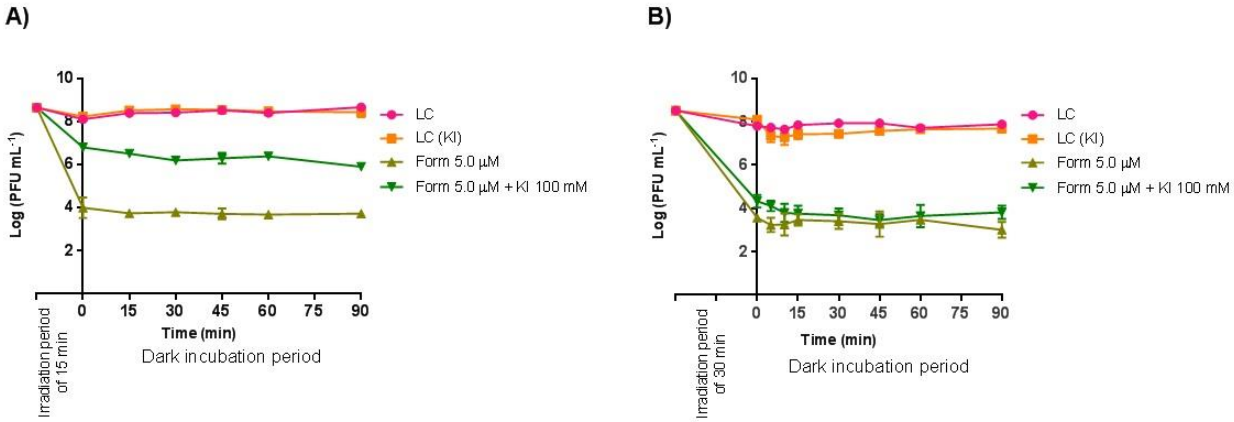

Figure 6. Effect of residual iodine species in dark incubation after aPDT assay on T4-like bacteriophage in filtered wastewater (0.45 µm filtration) with Form at 5.0 µM and KI at 100 mM, during 15 (A) and 30 (B) min of irradiation with white light (50 mW cm^{-2}). The values are expressed as the mean of three independent experiments; error bars represent the standard deviation (SD) between the experiments. In some cases, SD bars are covered behind the symbols.

In all the experiments, both light [LC and LC (KI)] and dark controls [DC and DC (Form+KI)] remained constant along the experiment period, meaning that Form + KI in the dark or KI in the presence of light have no toxic effect in the bacteriophage particles and that the white light radiation alone has no effect on the viral particles viability.

3.3 aPDT Assays in Non-filtered Wastewater

In Figure 7 are shown the effects of aPDT with Form at 10 μM with and without the potentiator H_2O_2 at concentrations of 2, 5 and 9% (Figure 7A) as well as the effects. After the results obtained from these assays at different concentrations, for Form at 5.0 μM the H_2O_2 effect was evaluated only at 5% (Figure 7B).

For the assays with Form at 10 μM without and with the addition of H_2O_2 at 2, 5 and 9% the addiction of H_2O_2 brought a significant improvement to the aPDT treatment comparing with the samples where only Form was used (*p* value < 0.0001). Moreover, when H_2O_2 was added to Form at 5 and 9% a significant increase in the phage inactivation was observed when compared to the samples where H_2O_2 was added at 2% (*p* value < 0.0001). The detection limit of the phage (more than 7 log PFU mL^{-1}) was reached just after 5 min of treatment (15 J cm^{-2} light dose) with Form and 5 and 9% H_2O_2.

For experiments with Form at 5.0 μM in combination with H_2O_2 at 5% a significant increase in phage inactivation was observed, enhancing the inactivation efficiency in ca. 4 log PFU mL^{-1} comparing with Form alone (after 15 min of treatment, 45 J cm^{-2} light dose), reaching the detection limit of more than 7 log PFU mL^{-1}, after just 5 min of treatment (15 J cm^{-2} light dose) (*p* value < 0.0001).

In the cases of the light [LC (H_2O_2)] dark controls [DC (Form+H_2O_2)] no decrease in phage concentration was detected. These results indicate that the viability of this bacteriophage was not affected by irradiation, nor by the presence of the H_2O_2 or by any of the tested combinations of Form plus H_2O_2 in the dark.

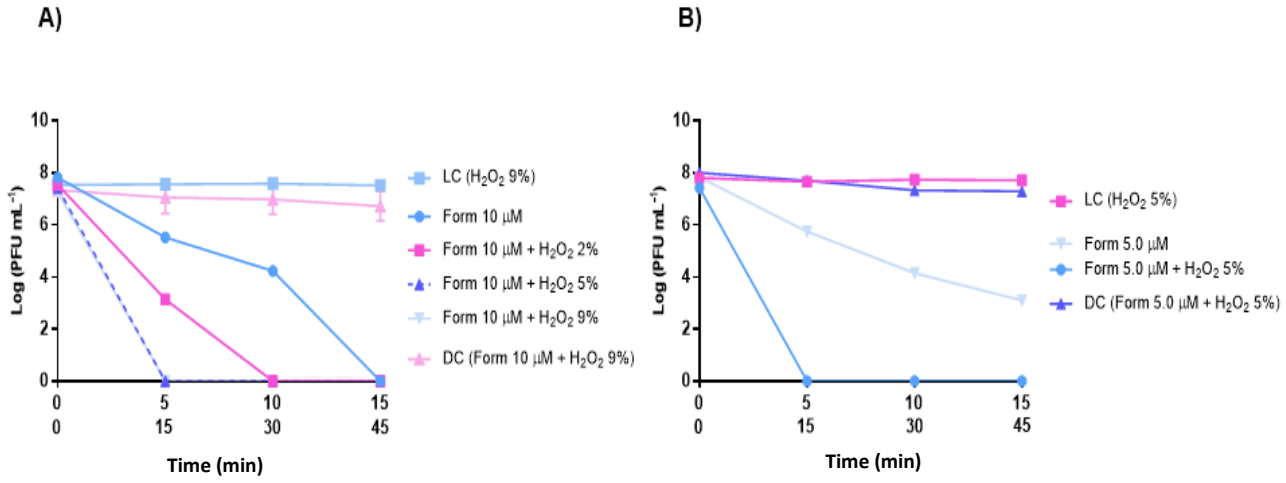

Figure 7. Inactivation of T4-like bacteriophage during aPDT, in non-filtered wastewater with Form at 10 µM alone and in combination with H_2O_2 (2, 5, and 9%) (A) and with Form at 5.0 µM alone and in combination with H_2O_2 (at 5%) (B), for 15 min of irradiation with white light (50 mW cm^{-2}). The values are expressed as the mean of three independent experiments; error bars represent the standard deviation (SD) between the experiments. In some cases, SD bars are covered behind the symbols.

4. DISCUSSION AND CONCLUSIONS

The photoinactivation of viruses occurs due to the generation of ROS, which interacts with components of the viral particles through oxidation reactions, as well as cleavage and cross-linking modifications, among others, leading to damages in fundamental structural and functional molecules (Costa et al. 2014). In general, molecular structures as the envelope lipids and proteins, the proteins of the capsid and the nucleic acids are known to be targets of the aPDT in viruses (Costa *et al.* 2014). Still, the efficiency of the process has shown to be highly dependent on some factors as the number and position of charges of the porphyrins and the composition of the substituents in the *meso*-positions of the porphyrin macrocycle (Costa *et al.* 2008). However, the efficiency of aPDT in more complex matrices can be also affected by the presence of particulate and dissolved organic matter, chemicals among other factors (Almeida *et al.* 2014; Bartolomeu *et al.* 2017; Filipe *et al.* 2017).

According to the literature, ROS are known to have a short life span due to their unstable electronic configuration, which consequently leads to a small diffusion range capacity. However, their diffusion range is also highly dependent on the type of environment. Since there is a large amount of organic matter/compounds in wastewater, it may allow the appearance of different microenvironments with different diffusion rates (Alves *et al.* 2014). Thus, the presence of compounds capable of interacting with ROS may affect the bacteriophage inactivation, since ROS will interact not only with the bacteriophage but also with the present compounds, and, consequently, be responsible for the observed differences in the bacteriophage T4-like photoinactivation rates between assays performed in PBS and filtered and non-filtered wastewater.

Some previous studies (Alves *et al.* 2011 and Arrojado *et al.* 2011), using Tri-Py(+)-Me (one of the PSs included in the Form used in the present work) as PS against several bacteria, had shown that aPDT efficiency was higher when performed in PBS comparing with its effectiveness in aquaculture wastewater. However, in this study when aPDT was done in wastewater (filtered and non-filtered), the bacteriophage inactivation was more effective than when aPDT was performed in PBS. The inactivation to the detection limit was reached after 15 min (45 J cm^{-2} light dose) in wastewater and only after 30 min (90 J cm^{-2} light dose) in PBS. In fact, in a more recent study, Almeida *et al.* (2014) performed aPDT against bacteria in hospital wastewater, resulting in a higher inactivation efficiency (during the initial period of treatment of 30 min, corresponding to 7.2 J cm^{-2} light dose) in the hospital wastewater when compared with PBS (a difference of ca. 2 log colony-forming unit per mL). Also, as previously suggested

by Almeida *et al.* (2014) and Bartolomeu *et al.* (2017), suspended and dissolved organic matter present in the aqueous matrix may act as an aPDT efficiency enhancer, influencing positively the PS activity, possibly by the presence of other compounds that may be found in wastewater, as pharmaceutical compounds and detergents. Likewise, Almeida *et al.* (2014) and Bartolomeu *et al.* (2017) justify this behavior due to the presence of organic matter that can act as a factor that influences the efficiency of the PS in aPDT process. However, in this study and other studies (Almeida *et al.* 2014; Bartolomeu *et al.* 2017) since no changes in bacteriophage viability were observed neither in the light and dark controls, it can be inferred that these dissolved compounds do not affect directly the viability of the bacteriophage.

In this study, when the content of suspended organic matter in the aqueous matrix was reduced, the aPDT efficiency had a significant increase. About 8 log PFU mL^{-1} were inactivated after 5 min of treatment (15 J cm^{-2} light dose) in the samples using 0.22 and 0.33 µm filtered wastewater when compared with the non-filtered wastewater samples, where about 8 log PFU mL^{-1} were inactivated only after 15 min of treatment (45 J cm^{-2} light dose). These results were also found in similar studies performed (Bartolomeu *et al.* 2017) where the presence of organic matter may have interfered with the efficiency of photoinactivation, once by filtration most of the particulate organic matter is removed. However, pharmaceuticals and personal care products are retained. This can also affect the effectiveness of aPDT by the combination of Form + KI, as the organic matter may act as a barrier to KI performance, leading to a delay over the treatment efficiency.

When aPDT was performed in filtered wastewater (by 0.45 µm), the minimum Form concentration that allowed significant inactivation (within the several concentrations tested, between 1.0 and 10 µM) was 3.0 µM. With this concentration, the detection limit of the method (reduction of 8 log PFU mL^{-1}) was reached after 30 min of irradiation (90 J cm^{-2} light dose), against 15 min of treatment when Form at 10 µM was used. When aPDT was performed in the presence of the potentiator KI (at 100 mM), no potentiation was observed. The inactivation in the samples with Form + KI was reached at the same time or even later when compared with the samples at the same Form concentration but without KI. These results are not in accordance with some previous studies where it is observed that KI enhances PDI efficiency for Gram-negative and Gram-positive bacteria, viruses, and fungi (Vieira *et al.* 2018), either when aPDT occurs via type I or by type II mechanisms, increasing the efficiency of bacterial inactivation. However, in a more recent study, conducted by the same authors, both effects were demonstrated – the potentiation and non-potentiation effect of KI in microorganisms inactivation. The potentiator effect of the KI was demonstrated against both Gram-positive (*S. aureus*) and Gram-negative bacteria (*E. coli*) and a fungus (*C. albicans*), with several Form concentrations (between 0.5 and 5.0 µM) (Vieira *et al.* 2019). However, the effect of Form with the addition of KI has not increased the aPDT efficiency of the T4-like bacteriophage, with PBS as the used aqueous matrix (Vieira *et al.* 2019). Vieira *et al.* (2019) showed that even at different Form concentrations (between 0.5 to 2.5 µM) with the addition of KI (at 100 mM), the bacteriophage inactivation occurred at the same time or even later when compared with the results obtained in the samples where just the Form was added. However, the authors also showed that with the lowest Form concentration tested (0.1 µM), the aPDT efficiency was

higher when the KI was added as a potentiator, reaching the detection limit after 20 min of treatment (3.0 J cm^{-2} light dose), against no total inactivation after 45 min (6.75 J cm^{-2} light dose) when just Form was added to the sample (Vieira *et al.* 2019). The authors suggested that this effect might be due to the fast inactivation by the ROS formed through the Form activation, and by that, not giving time to the iodide reactive species (through the combination of Form+KI) to be formed (Vieira *et al.* 2019). And so, justifying the shown potentiator effect of the KI when Form was used at low concentrations, once the formation of ROS by Form would be slower due to the low Form concentration, allowing the iodide species to be formed and act. This enlightenment might be the explanation by our results as well, adding to the fact that our experiments were performed in filtered wastewater and so, more effect of external factors and entropy was added to the system and, therefore, the iodide reactive species may dissipate and do not act with all its efficiency directly on the bacteriophage inactivation. The possible combination of these effects might as well be the reason why during dark incubation after irradiation treatment, the effect of the iodide reactive species did not show any effect on the bacteriophage inactivation.

With the purpose of mimic the scenario close to the reality of a WWTP, non-filtered wastewater was used. As in this case, all the organic matter, chemical compounds and various microorganisms are present, the Form concentration was increased to 10 µM. After 15 min of light exposure (45 J cm^{-2} light dose), the bacteriophage decreases of ca. 8 log PFU mL^{-1}, inactivation to the detection limit of the method, was reached. These results were very similar to those obtained in 0.45 µm filtered wastewater and better than those obtained in PBS, which reveals that the organic matter present in both matrices must be relevant for the inactivation of the bacteriophage. Our results are in accordance with previous experiments performed in hospital wastewater (Almeida *et al.* 2014), in which PDI efficiency was higher in the wastewater matrix compared to PBS. In our study, the bacteriophage inactivation to the detection limit of the method was achieved after just 15 min (45 J cm^{-2} light dose) when wastewater was used, but only after 30 min of treatment (90 J cm^{-2} light dose) when PBS was used. As suggested for the hospital wastewater study (Almeida *et al.* 2014), in our case the presence of organic matter and chemical compounds seems to increase the effectiveness of aPDT.

With the aim of searching for an alternative to improve the efficiency of aPDT in non-filtered wastewater, experiments were conducted with a combination of Form and H_2O_2. The compound H_2O_2 is commercialized at concentrations of 3 and 9%. Thus, tests were performed with concentrations equal to and lower than 9% to be in accordance with what is recommended for human use to prevent any risk of toxicity (PHE, 2009). For the tested concentrations of 2, 5 and 9% combined with Form at 10 µM, 5% of H_2O_2 was the lowest concentration with the best performance for the combination of Form + H_2O_2. A reduction to the detection limit of the method, reduction of about 7.5 log PFU mL^{-1}, was reached after 5 min (15 J cm^{-2} light dose) of treatment. Thus, it was possible to reduce the time treatment (in 10 min) of non-filtered wastewater relatively to the treatment with the same concentration of Form alone. Additionally, when Form was used at half concentration, 5.0 µM, with the addition of H_2O_2 at 5%, the efficiency of the bacteriophage inactivation was the same with Form at 10 µM plus the H_2O_2 at 5 and 9%, showing that the addition of H_2O_2 to the system brings a huge improvement to the aPDT

effectiveness in the inactivation of the bacteriophage, improving the action of the PS Form. Parallelly, in light and dark controls, containing H_2O_2 at the highest concentration tested (9%), no effects on the bacteriophage viability were detected, showing that the H_2O_2 alone, at the concentration tested, does not promote bacteriophage inactivation. As previously mentioned by Awad *et al.* (2013), H_2O_2 alters the microbial external structures permeability. This effect might allow the PS accumulation in the viral particles. Withal, the presence of H_2O_2 might also increase the molecular oxygen availability and, consequently, increasing the ROS formation (Garcez *et al.* 2011; Awad *et al.* 2013).

Overall, photodynamic therapy with Form even used alone, showed to be efficient against viruses inactivation in wastewater. The addition of H_2O_2 during aPDT with the Form potentiate the bacteriophage inactivation process, allowing to reduce the PS concentration and the treatment time. The results obtained in this study increase the support of aPDT application as an alternative approach to the actual commonly used methods of water disinfection, ensuring the safety of tertiarily treated wastewater. This alternative approach would help to surpass the disadvantages of chlorine use as one of the most used methods of water disinfection, namely the formation of by-products with potential health risk as a consequence of its reaction with organic compounds. Such performance was also proven when combining Form with the potentiator H_2O_2. Additionally, future studies must be done in order to improve the knowledge about the possible effect of the organic matter in aPDT efficiency.

CHAPTER 3 – GENERAL CONCLUSIONS AND FUTURE WORK

3.1) CONCLUSION

In this study, Form shown to be effective in the photoinactivation of bacteriophage T4-like, in domestic-industrial WW. Form effectiveness against the bacteriophage in filtered WW was correlated with the PS concentration. When combined with KI, Form was less effective to inactivate the bacteriophage. With the increase of organic matter, it was observed a significant decrease in the efficiency of Form. Form alone proved to be an efficient PS to photoinactivate the bacteriophage in non-filtered WW, however, the presence of H_2O_2 enhanced the photodynamic effect. In conclusion, Form can be an effective alternative to control viruses in WW, particularly if combined with H_2O_2.

3.2) FUTURE WORKS

Future studies should focus on:

- The chemical characterization of water matrices before and after each test to assess existing biological entities, organic matter and chemical compounds and thus allowing to perceive the influence of their effect on PDI;
- To test PDI efficiency in different types of wastewater;
- To test lower Form concentrations with H_2O_2 to develop the knowledge of their potential combined effect;
- To test PDI efficiency with Form alone and in combination with H_2O_2 at different levels of wastewater turbidity;
- Evaluate the impact of PDI with Form alone and in combination with H_2O_2 in the water community;
- To perform these photoinactivation assays under natural sunlight conditions and compare the obtained results with those performed with artificial light;
- To test the effectiveness of this disinfection approach on other bacteriophages, individually and in cocktails.

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