Universidade de Aveiro

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MARTA SILVA DE CARVALHO GOMES

Desinfeção de águas residuais na mitigação do SARS-CoV-2 por tratamento fotodinâmico

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia realizada sob a orientação científica da Doutora Maria Adelaide de Pinho Almeida, Professora Catedrática do Departamento de Biologia da Universidade de Aveiro, e coorientação da Doutora Ana Teresa Peixoto de Campos Gomes, Investigadora Auxiliar do Centro de Investigação Interdisciplinar em Saúde da Universidade Católica Portuguesa.

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Palavras-chave

Pseudomonas syringae, inativação fotodinâmica (PDI), fatores ambientais, porfirina, água residual, fago ϕ6.

Resumo

Desde dezembro de 2019 que o mundo se tem deparado com uma pandemia causada pelo SARS-CoV-2. Apesar da maioria dos pacientes apresentar sintomas ao nível do sistema respiratório, o vírus também está presente no trato intestinal. É então perentória a necessidade de perceber a sobrevivência deste novo vírus em águas residuais (AR) em diferentes condições ambientais, bem como, de estudar formas eficientes e seguras para inativar o SARS-CoV-2 nas AR, uma vez que os métodos usados atualmente podem levar à formação de compostos tóxicos, sendo dispendiosos e pouco eficientes. Como tal, a inativação fotodinâmica microbiana (IF) usando fotossensibilizadores, oxigénio e luz visível, deve ser tida em conta, visto já ter dado provas da sua eficiência contra vários microrganismos, nomeadamente, vírus. Neste estudo, o fago ϕ6 foi usado como modelo do vírus SARS-CoV-2. Foi avaliada a viabilidade do fago em AR, a sua inativação por IF e o impacto da AR tratada por IF sobre os microrganismos aquáticos. Os resultados dos testes de viabilidade do fago ϕ6 indicam que este se mantém viável em AR durante: (1) 84 dias a 17 ºC, 35 dias a 25 ºC e 24 horas a 37 ºC; (2) 63 dias em AR com pH 8 e pH 9, e pelo menos 84 dias a pH 6; (3) pelo menos 84 dias quando o valor de salinidade das águas é de 15 g kg^{-1} e 34 g kg^{-1}; (4) 24 horas quando irradiado por luz UV-B; e (5) 4 horas quando exposto a radiação solar. A IF, usando como fotossensibilizador a TetraPy(+)Me e luz branca artificial a 50 mW cm^{-2}, provou ser eficiente na inativação do fago ϕ6, ao fim de 10 minutos de tratamento em PBS e 30 minutos em AR. Quando a proporção de efluente e AR previamente tratada por IF é superior a 1:2, não foi verificada toxicidade nos microrganismos marinhos aquáticos nativos. Este trabalho mostrou que o ϕ6 se mantém viável em diferentes ambientes por um período de tempo considerável, sendo a IF uma abordagem eficiente para a sua inativação, não afetando os microrganismos nativos do meio ambiente marinho onde as AR são libertadas após tratamento.

Keywords

Pseudomonas syringae, photodynamic inactivation (PDI), environmental factors, porphyrin, wastewater, phage ϕ6

Abstract

Since December 2019, the world has faced a pandemic caused by SARS-CoV-2. Although most patients present symptoms at the level of the respiratory system, it is already known that this virus is also present in the intestinal tract. As such, transmissions via the fecal-oral or fecal-nasal route cannot be excluded as possibilities. It is therefore imperative to understand the survival of this new virus in wastewater (WW) under different environmental conditions, as well as to study efficient and safe ways to inactivate SARS-CoV-2 in water, as the methods currently used can lead to the formation of toxic compounds, be expensive or ineffiicient. As such, photodynamic inactivation (PDI) with photosensitizers, oxygen and visible light should be taken into account, as it has already proven to be efficient against several microorganisms, namely viruses. In this work the phage ϕ6 was used as a model of virus SARS-CoV-2. The viability of phage in WW, its inactivation by PDI and the impact of PDI-treated WW on aquatic microorganisms were evaluated. The results of the phage ϕ6 viability tests indicate that it remains viable in WW for: (1) 84 days at 17 ºC, 35 days at 25 ºC and 24 hours at 37 ºC; (2) 63 days when the pH of the water was adjusted to 8 and 9 and at least 84 days at pH 6; (3) at least 84 days when the water salinity value is 15 g kg^{-1} and 34 g kg^{-1}; (4) 24 hours when irradiated by UV-B light; and (5) 4 hours when exposed to solar radiation. PDI, using TetraPy(+)Me as photosensitizer and artificial white light at 50 mW cm^{-2}, proved to be efficient to inactivate phage ϕ6 after 10 minutes of treatment in PBS and 30 minutes in WW. When the proportion of WW previously treated by PDI is greater than 1:2, no negative effects were observed in native aquatic marine microorganisms. The results of this work showed that phage ϕ6 remains viable in different environments for a considerable amount of time, with PDI being an efficient approach for its inactivation, not affecting native microorganisms in the marine environment where WW are released after treatment.

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List of Acronyms

PDI, Photodynamic inactivation

CoVs, Coronaviruses

SARS, Severe Acute Respiratory Syndrome

MERS, Middle East Respiratory Syndrome

SARS-CoV-2, Severe Acute Respiratory Syndrome Coronavirus 2

WHO, World Health Organization

COVID-19, Coronavirus Disease 2019

ORFs, Open-Reading Frames

S, Spike Glycoprotein

RBD, Receptor Binding Domain

M, Matrix

E, Envelope

N, Nucleocapsid

ACE2, Angiotensin-Converting Enzyme 2

WWTPs, Wastewater Treatment Plants

PS, Photosensitizer

ROS, Reactive Oxygen Species

O_2, Molecular Oxygen

$^1O_2$, Singlet Oxygen

Tetra-Py(+)-Me, 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide

CFUs, Colony Forming Units

WW, Wastewater

PBS, Phosphate Buffered Saline

TSB, Tryptic Soy Broth

TSA, Tryptic Soy Agar

PCA, Plate Count Agar

Chapter 1

1. Introduction

1.1. Objectives

The main objective of this work was to evaluate the potential application of photodynamic inactivation (PDI) for the inactivation of SARS-CoV-2 in wastewater, using non-toxic and already approved compounds under artificial light. The phage viability under different conditions of temperature, pH, salinity, solar and UV-B radiation was also evaluated. The effect of the treated effluents with PDI on the viability of native marine aquatic microorganisms was also determined, in order to evaluate the safety of the discharge of the treated effluents in the marine environment, where WW is normally discharged.

1.2. Guide of document organization

The present document is structured in three chapters, which are summarized as follows:

Chapter 1. Introduction. This chapter intends to frame the theme of the work: the context of the problem, the motivations to develop this work and the main objectives we intend to reach.

Chapter 2. State of the art. In this topic are presented generalities about the basic concepts of the theme of this work, including information about the microorganism and the therapeutic approach.

Chapter 3. Disinfection of wastewater in the mitigation of SARS-CoV-2 by photodynamic treatment. This chapter is structured as an original research manuscript, including a brief introduction to the topic, the description of the used materials and methods; the obtained results are here shown and discussed.

Chapter 2

2. State of the Art

2.1. SARS-CoV-2 pandemic

In the last two decades, coronaviruses (CoVs) have already been the cause of two epidemics, Severe Acute Respiratory Syndrome (SARS) in 2002 and 2003 (Drosten et al., 2003; Ksiazek et al., 2003) and Middle East Respiratory Syndrome (MERS) in 2012 (Zaki et al., 2012; Zumla et al., 2015). CoVs are a diverse group of viruses that infect different animals and can cause respiratory infections, from mild to severe, in humans (Hu et al., 2021). After these epidemic events, there were several signs of possible future disease outbreaks: CoVs undergo genetic recombination (Woo et al., 2006); the presence of a large reservoir of SARS-linked coronaviruses in horseshoe bats in China (Ge et al., 2013; W. Li et al., 2005); and the fact that previous studies have demonstrated the ability of some SARS-CoVs from bats to infect humans (Cheng et al., 2007; Cui et al., 2019; Lau et al., 2005; Wang et al., 2018). As expected, at the end of 2019 there was a new outbreak of CoVs that spread rapidly around the world and that surpassed SARS and MERS in terms of numbers of infected people and spatial amplitude of epidemic areas due to its transmissibility and which represents a threat to global public health (Hu et al., 2021).

The virus SARS-CoV-2 was first detected in Wuhan, China, and has spread rapidly since December 2019, causing more than 10,000 confirmed infections and 4,000 deaths from that date to March 10, 2020 (Neher et al., 2020). On January 30, the spread of SARS-CoV-2 was declared a public health emergency of international concern by the World Health Organization (WHO) (Neher et al., 2020). On February 11, 2020, the International Committee on Virus Taxonomy and WHO announced the official name of the virus, SARS-CoV-2, and of the 2019 coronavirus disease, COVID-19, respectively (Neher et al., 2020). On March 11, 2020, WHO declared the outbreak of the new coronavirus (SARS-CoV-2) as a pandemic (Neher et al., 2020).

As of April 6, 2020, 1285257 cases of COVID-19 had been recorded worldwide with a mortality rate of approximately 5.4% (Tu et al., 2020).

Regarding the epidemiological characteristics of COVID-19: the majority of confirmed positive cases, 86.6%, are aged between 30 and 79 years; most deaths occurred in patients aged ≥60 years, with the highest fatality rate, 20.3%, in the range ≥80 years; in patients classified as critical cases, lethality reached 49% (Chen et al., 2020). The most common symptoms of COVID-19 include fever (83-98%), cough (59-82%), shortness of breath (19-55%) and muscle pain (11-44%)(Huang et al., 2020). Evidence shows that infected cases can transmit the virus before the onset of symptoms and even after treatment of pneumonia by COVID-19, justifying the predominance of human-to-human transmission in communities and between members of the same family (Tu et al., 2020). It is mainly transmitted by droplets and aerosols from asymptomatic and symptomatic infected individuals (C. wei Lu et al., 2020). Indirect contact through contaminated surfaces can also be another cause of infections (Lauxmann et al., 2020).

According to the Johns Hopkins University Center for System Science and Engineering, as of August 11, 2020, more than 20 million cases of COVID-19 and more than 733,000 deaths in 216 countries on six continents have been confirmed (Dong et al., 2020). The high mortality was mainly due to the overload of health resources(Hu et al., 2021).

Presently, there have been 243 572 402 confirmed cases of COVID-19, including 4 948 434 deaths, reported to WHO, all around the world (WHO, 2021).

2.1.1. SARS-CoV-2

SARS-CoV -2 belongs to the *Coronaviridae* family (Tu et al., 2020). This family is characterized by containing an enveloped, single-stranded RNA genome. They are the largest known RNA viruses, have genomes ranging between 25 and 32 kb and a virion of 118-136 nm in diameter. The family is divided into two subfamilies, *Coronavirinae* and *Torovirinae* (Paz & Ruíz, 2017). Members of the subfamily *Coronavirinae* are common among mammals, more than 60 CoVs have been isolated from bats and most belong to the genus betacoronavirus. Bats have a long history of coevolution with this virus. Until 2002, CoVs were not considered major human pathogens, however this has changed with the outbreak of SARS-CoV and MERS-CoV and now with that of SARS-CoV-2 (Paz & Ruíz, 2017).

The first complete genome of the SARS-CoV-2 virus reported is 29.9 kb (F. Wu et al., 2020). The genome has a highly conserved zone that is common to other CoVs, consisting of six major open-reading frames (ORFs), and a set of other accessory genes (de Groot et al., 2013). Four ORFs of SARS-CoV-2 genome encode four essential structural proteins: (1) spike (S) glycoprotein (S1 and S2 subunits) that attaches to the host receptor through the receptor binding domain (RBD) (S1 subunit), determines the virus host range (S1 subunit), and mediates virus-cell membrane fusion (S2 subunit); (2) matrix (M) protein that mediates nutrients transport across the transmembrane, bud release and envelope formation; (3) small envelope (E) protein; and (4) nucleocapsid (N) protein which interfere with the host innate immune response (A. Wu et al., 2020). The CoVs peak glycoprotein constitutes the main neutralizing target for antibodies after infection, and therefore, is a focus for vaccine design, as it mediates the entry of the virus genome into host cells (Tortorici & Veesler, 2019).

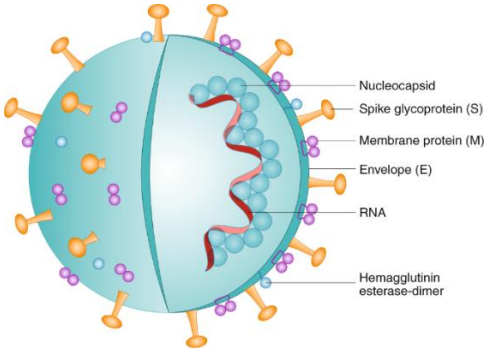

Figure 1: Schematic representation of SARS-CoV-2 struture (Florindo et al., 2020).

The host receptor used by SARS-CoV-2 is the same one used by SARS-CoV to infect humans, angiotensin-converting enzyme 2 (ACE2) (Zhou et al., 2020). SARS-CoV-2 also has an receptor binding domain that binds with high affinity to ACE2 from humans and other species with high receptor homology, explaining its wide host range (Hu et al., 2021).

2.1.2. SARS-CoV-2 in wastewater

Since SARS-CoV-2 are excreted in the urine and feces of infected individuals, regardless of the severity of symptoms, these viruses can be found in wastewater treatment plants (WWTPs) (Heller et al., 2020). The presence of SARS-CoV-2 in sewage samples is already confirmed by studies from different countries, namely in Netherlands (Medema et al., 2020); Wuhan, China (Zhang et al., 2020); EUA (Lodder & de Roda Husman, 2020); Australia (Wurtzer et al., 2020); Italy (Ahmed et al., 2020); between others.

Although virus detection in wastewater is beneficial in terms of early warning in epidemiological terms (Arslan et al., 2020), it can also pose a risk to public health, as incomplete removal of the virus by WWTPs can lead to outbreaks if individuals are exposed by inhalation or aerosol ingestion (Arslan et al., 2020). These risks vary in different societies, depending on the level of control measures, environmental conditions and facilities. As such, in developing societies the challenge can be greater. Having inefficient health systems leads to more outbreaks, increasing the viral load in wastewater, putting pressure on WWTPs, which in turn are also not equipped with the necessary technology to effectively remove the virus, which can result in frequent, recurrent or periodic post-pandemic outbreaks (Arslan et al., 2020), as previously reported for other viral diseases, notably during the SARS outbreak in 2003 (Usman et al., 2020).

Even in countries with the most efficient wastewater treatments, treatment plants produce a large amount of solid sludge that can carry various viruses, including SARS-CoV-2 (K. Xiao et al., 2019), and in higher concentrations than those found in raw wastewater (Ahmed et al., 2020; F. Xiao et al., 2020). Although in some communities there are already sludge treatments to reduce the load of microorganisms, it is still unknown whether these treatments can efficiently inactivate SARS-CoV-2 (Arslan et al., 2020).

According to the 2017 United Nations World Water Development Report, 80% of wastewater is released into the environment without proper treatment worldwide (Usman et al., 2020). All of this evidence shows the need to consider potential risks to developing communities, as well as the need for information on the effectiveness of current wastewater disinfection treatments (Usman et al., 2020).

2.2. Photodynamic Inactivation

2.2.1. Photodynamic Inactivation Principles

Light has been used in the treatment of diseases since antiquity. However, it was not until the beginning of the 20th century that Tappeiner and Jodlbauer defined the photodynamic effect concept

(Ackroyd et al., 2001). The essential elements for this therapy are (i) the presence of a photosensitizer (PS), (ii) light of a suitable wavelength to match the PS absorption spectrum and (iii) molecular oxygen (Guillemin et al., 1999). The basic principle of PDI is the energy absorption in the form of light by the PS, which reach an excited state and react with ambient oxygen, leading to the formation of reactive oxygen species (ROS) (Dai et al., 2012).

PDI is a selective therapy, since it requires the presence of a PS and the PS by itself is not toxic, allowing to target PS to unwanted cells or tissues and limiting the effect to regions where light of the proper wavelength is applied (Dai et al., 2012).

PDI has proven to be effective in destroying microorganisms, namely Gram-positive and Gram-negative bacteria as well as viruses, fungi and parasites (Carvalho et al., 2007; Costa et al., 2008, 2012). The main targets of PDI are microbial external structures, which are irreversible damaged after treatment (Alves et al., 2014).

The interaction between agents that participate in the PDI process can occur through two distinct pathways: type I and type II mechanisms. In the type I mechanism, the interaction runs between the excited PS and the substrate leading to the formation of radical species or peroxides, while in the type II mechanism, the interaction occurs between excited PS and molecular oxygen (O_2), forming singlet oxygen ($^1O_2$) (Wainwright & Crossley, 2004).

2.2.2. Photosensitizer

The first substance to be used as a microbial photodynamic agent was acridine orange. Currently, PS with cyclic tetrapyrrolic structures such as porphyrins, chlorins, bacteriochlorins, and phthalocyanines are among the most used in PDI (Benov, 2015). Porphyrins, chlorins and bacteriochlorins have maximum light absorption in the red portion of the electromagnetic spectrum, which allows for deeper tissue penetration (Macdonald & Dougherty, 2001).

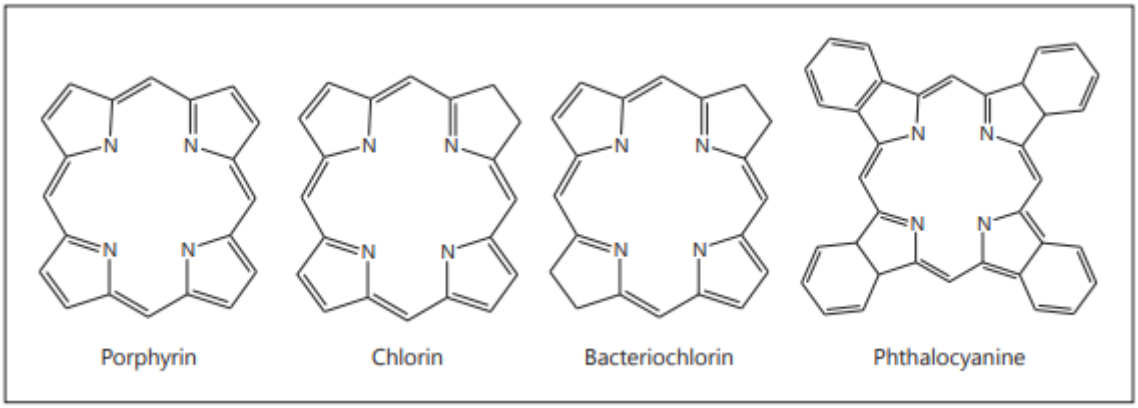

Figure 2: Main groups of PSs used in PDT (Benov, 2015).

Porphyrins have absorption bands in the visible region of the electromagnetic spectrum, with the highest intensity band in the 400 nm region (Soret band) and less intense absorption bands with

wavelengths between 500 and 600 nm (Q bands) (Wainwright & Crossley, 2004). They are aromatic heterocycles made up of four pyrrole-type units linked together by methine bridges.

The tetracationic porphyrin derivative TetraPy(+)Me (5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide) as PS has the advantages of easy accessibility and proven efficiency in inactivating Gram-positive and Gram-negative bacteria, as well as other microorganisms such as viruses (Bartolomeu et al., 2017).

2.2.3. Photodynamic treatment in the inactivation of phage ϕ6

The use of photodynamic treatment has already been approved for the treatment of cancer, but also for non-oncological situations (Almeida et al., 2020). Photodynamic treatment has also proven effective in inactivating all types of microorganisms. As such, the use of photodynamic treatment deserves to be considered as an alternative approach against a SARS-CoV-2.

In the case of WW, and since PDI is truly antimicrobial, it is also expected to be effective against other microorganisms, namely SARS-CoV-2 (Almeida et al., 2020). PDI seems to be a good approach for the treatment of WW, as it has already shown promising results in the inactivation of other viruses, namely enveloped viruses, such as SARS-CoV-2 (Costa et al., 2011, 2012).

PDI may still be a good solution for SARS-CoV-2 inactivation under other conditions. PDI under artificial white light and sunlight can also be used to disinfect surfaces and fabrics (Almeida et al., 2020).

Since most of the laboratories do not have the necessary security level to carry out experiments with SARS-CoV-2, the phage ϕ6 has been used as a model of SARS-CoV-2, since it was considered as a good substitute for enveloped RNA viruses such as CoVs (C. D. Lytle et al., 1991).

The bacteriophage ϕ6 has been used as a model of pathogenic human viruses in surface inactivation studies (Aquino De Carvalho et al., 2017). Recent studies have used this bacteriophage as a model for SARS-CoV-2 (virus responsible for the COVID-19 pandemic) to assess its persistence on porous and non-porous surfaces (Whitworth et al., 2020b) and its survival in droplets dispersed on glass surfaces (Fedorenko et al., 2020). The phage ϕ6 has also recently been used as a model for the coronavirus in ultraviolet light surface disinfection studies (Ma et al., 2021).

The disinfection of potentially contaminated products is already carried out using chemical disinfectants, heat and ultraviolet irradiation (Kratzel et al., 2020; Vatter et al., 2020). However these

techniques have already proven to be harmful to sensitive materials and cells, namely human cells (Vatter et al., 2020).

Few studies have been done so far about the photoinactivation of phage ϕ6. (Vatter et al., 2020) studied the effect of visible violet light with a wavelength of 405 nm for the inactivation of phage ϕ6 in two aqueous solutions without the addition of photosensitizers, showing a reduction to three logarithmic levels of colony forming units (CFUs) (Vatter et al., 2020). To date, no study has been done on the inactivating effect of visible light on coronaviruses, apart from an “accidental” study in 1965 in which Cartwright et al. found a two-log reduction of the virus after accidental exposure of a medium containing coronavirus to sunlight for one day (Cartwright et al., 1965).

Almeida et al (2020) also suggest the use of PDI to inactivate SARS-CoV-2 in WW (Almeida et al., 2020).

Chapter 3

Disinfection of wastewater in the mitigation of SARS-CoV-2 by photodynamic treatment

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Keywords: *Pseudomonas syringae*, photodynamic inactivation (PDI), environmental factors, porphyrin, wastewater, phage ϕ6

Abstract

The last few years have been marked by the SARS-CoV-2 pandemic. This virus is found in the intestinal tract reaching the wastewater system, and consequently the natural receiving water bodies. As such, inefficiently treated wastewater (WW) can be a means of contamination. The disinfection methods of WW currently used can lead to the formation of toxic compounds, be expensive, or inefficient. As such, new alternative approaches must be considered, namely microbial photodynamic inactivation (PDI). In this work, the phage ϕ6, which has been used as a suitable model for enveloped RNA viruses such as coronaviruses (CoVs), was used as a model of the SARS-CoV-2. Firstly, to understand the survival of the virus in the environment, phage ϕ6 was subjected to different laboratory-controlled environmental conditions (temperature, pH, salinity, and solar and UV-B irradiation) and its persistence over time was assessed. Second, to assess the efficiency of virus inactivation, PDI assays were performed, both in phosphate-buffered saline (PBS), a well-known composition aqueous matrix, and in a real WW matrice, secondarily treated WW. Third, as, in general, WW is discharged into the marine environment after treatment, the safety of PDI-treated WW was assessed through the determination of the viability of native marine water microorganisms after their contact with the PDI-treated effluent. Overall, the results showed that phage ϕ6, used as SARS-CoV-2 surrogate, remains viable in different environmental conditions for a considerable period of time, with PDI being an efficient approach in the inactivation of the virus, and with the PDI-treated effluent showing no toxicity to native aquatic microorganisms under dilution realistic conditions, endorsing PDI as an efficient and safe WW tertiary disinfection method. As the results

were obtained for phage ϕ6, that although is considered a suitable model of SARS-CoV-2, further studies using the SARS-CoV-2 are necessary.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a new beta-coronavirus, is the causative agent of the pandemic that began in December 2019 in Wuhan, China (Neher et al., 2020). It is an RNA virus, with a genome ranging between 25 and 32 kb, and a virion of 118-136 nm in diameter (Paz & Ruíz, 2017).

Along with respiratory infection, SARS-CoV-2 is also known to infect the gastrointestinal tract via the angiotensin-converting enzyme (ACE2) receptor that is expressed by epithelial cells in the gastrointestinal system (F. Xiao et al., 2020). Since the RNA of the virus has already been detected in the feces of infected individuals for prolonged periods of time the potential for SARS-CoV-2 to be transmitted via the fecal-oral (Yeo et al., 2020) or nasal-fecal pathways, is a source of concern (F. Xiao et al., 2020).

A large part of the WW released in the environment, around the world, is not adequately treated for the elimination of microorganisms (Usman et al., 2020). Even in WWTPs with adequate treatments, in general, WW are treated secondarily and released into rivers and sea waters, still containing high concentrations of microorganisms (Bartolomeu et al., 2017). These data highlight the potential risks of transmission of emerging microorganisms through WW discharge in the environment, which imply the need for the development of efficient WW disinfection treatments (Usman et al., 2020). Since SARS-CoV-2 is excreted in the feces of infected individuals, regardless of the severity of symptoms, these viruses can be found in WWTPs (Heller et al., 2020). The presence of SARS-CoV-2 in sewage samples was already confirmed by studies from different countries, namely in the Netherlands (Medema et al., 2020); Wuhan, China (Zhang et al., 2020); EUA (Lodder & de Roda Husman, 2020); Australia (Wurtzer et al., 2020); Italy (Ahmed et al., 2020); among others. It can also pose a risk to public health, as incomplete removal of the virus by WWTPs can lead to outbreaks if individuals are exposed to inhalation or aerosol ingestion (Arslan et al., 2020).

Tertiary disinfection treatments are already used, however, they can be expensive, toxic to aquatic organisms and induce genetic damage to microorganisms (Brown et al., 2006). As such, the development of new safe technologies for WW disinfection must be taken into account.

PDI with different photosensitizers and under visible light has already been shown to be effective in inactivating Gram-positive and Gram-negative bacteria, viruses, fungi and parasites (Alves et al., 2015; Carvalho et al., 2007; Costa et al., 2008, 2012; Jemli et al., 2002). PDI is a selective therapy that occurs only in the presence of a PS, light with an appropriate wavelength (Calin & Parasca, 2009) and molecular oxygen (Henderson & Dougherty, 1992). With the absorption of light, PS reaches the triplet state through a singlet excited state of short duration which allows it to have the

ability to transfer energy to O_2 giving rise to $^1O_2$, or to surrounding substrates giving rise to ROS (Calin & Parasca, 2009), that lead to the oxidation of microbial constituents.

The purpose of this study was to evaluate the potential application of PDI for the inactivation of SARS-CoV-2 in WW, using a non-toxic and already tested compound as photosensitizer, a tetracationic porphyrin (TetraPy(+)-Me), under artificial white light. TetraPy(+)-Me was chosen as PS due to its easy accessibility and recognized efficiency in inactivating microorganisms such as viruses (Bartolomeu et al., 2017). In the PDI assays, the phage ϕ6 was used as a SARS-CoV-2 surrogate. Phage ϕ6 is an enveloped dsRNA virus, with an RNA genome of 13.5 kbp and a size of 75 nm (Gonzalez et al., 1977), that has been previously used as a suitable model for enveloped RNA viruses such as coronaviruses (CoVs) (C. D. Lytle et al., 1991). The bacteriophage ϕ6 has been used as a model of pathogenic human viruses in surface inactivation studies (Aquino De Carvalho et al., 2017). Recent studies have used this bacteriophage as a model for SARS-CoV-2 to assess its persistence on porous and non-porous surfaces (Whitworth et al., 2020a) and its survival in droplets dispersed on glass surfaces (Fedorenko et al., 2020). The phage ϕ6 has also recently been used as a model for the coronavirus in ultraviolet light surface disinfection studies (Ma et al., 2021). Phage ϕ6 has been previously used as a good substitute for enveloped viruses such as CoVs, due to its tolerance to ultraviolet radiation inactivation (Ye et al., 2018), temperature and humidity (Prussin et al., 2018), as well as its recovery from hands (Casanova & Weaver, 2015) and persistence in water, sewage, and on surfaces (Aquino De Carvalho et al., 2017; Silverman & Boehm, 2020; Ye et al., 2016), are similar to those observed for CoVs. Furthermore, the phage has also been suggested as a substitute for enveloped human viruses in visible light photodynamic inactivation (C. D. Lytle et al., 1991).

2. Material and Methods

2.1. Experimental design

The tests were carried out with a surrogate model of SARS-CoV-2, the phage ϕ6. This phage is a member of the Cystoviridae family [32], it is an enveloped dsRNA virus, with an RNA genome of 13.5 kbp and a size of 75 nm [21], which multiplies in *Pseudomonas syringae* bacteria.

Firstly, to understand the survivability of the virus in the environment, we subjected phage ϕ6 to different laboratory-controlled environmental conditions (temperature, pH, salinity, and solar and UV-B irradiation) and assessed its persistence over time. The range of the tested values of temperature, pH, salinity and solar and UV-B irradiation were selected having into account the range of values observed during the year, and for temperature and pH it was considered the range of values for these variables in WW.

To evaluate the potential of PDI in the photo-inactivation of phage ϕ6 and to select the best photo-inactivation conditions to be used in WW disinfection, in vitro assays were performed: first in the well-known composition aqueous matrix phosphate-buffered saline (PBS), followed by the PDI assays on a real WW matrice, secondarily treated WW.

Once WW is discharged into the marine environment after treatment, the safety of PDI-treated WW was assessed through the determination of the viability of native marine water microorganisms after contact with the PDI-treated effluent.

2.2. Bacterial strain and growth conditions

Bacteria *Pseudomonas sp*. (DSM 21482) was used in this study as the phage host and was purchased from Leibniz-Institute DSMZ—Deutsche Sammlung von Mikroorganis-men und Zellkulturen GmmH (Braunschweig, Germany). The bacterial cells were culti-vated in Tryptic Soy Broth (TSB, Liofilchem, Roseto degli Abruzzi (TE), Italy) at 25 ˚C for 18 h under orbital shaking (120 rpm). Subsequently, bacterial glycerol stocks were done in 10% glycerol and stored at -80 °C. Before each assay, a bacterial stock was aseptically in-oculated into 30 mL of fresh TSB and incubated overnight as described above, until reaching a viable cell density of approximately 10^8 – 10^9 colony-forming units per mL (CFU mL^{-1}).

2.3. Preparation of phage ϕ6 and enrichment

Phage ϕ6 (DSM 21518) was purchased from Leibniz-Institute DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmmH (Braunschweig, Germany). Phage suspensions were obtained from a phage stock previously prepared in SM buffer [0.1 M NaCl (Sigma, St. Louis MO, USA), 8 mM $MgSO_4$ (Sigma, St. Louis MO, USA), 20 mM Tris-HCl (Sigma, St. Louis MO, USA), 2% (w/v) gelatin, pH 7.5)] using *Pseudomonas sp*. as the phage host. To 50 mL of SM buffer were added 2 mL of phage stock and 1 mL of *Pseudomonas sp*. in the exponential growth phase. The suspension was incubated overnight at 25 ˚C with orbital shaking (60 rpm). The preparation was centrifuged at 12,000 rpm and then filtered through a membrane with a pore size of 0.22 µm (Merck Millipore, Darmstadt, Germany), to remove intact bacteria or bacterial debris. The titer was determined by the double-layer agar method and the phage suspension was stored at 4 ˚C. Successive dilutions of the phage suspension were made in phosphate-buffered saline [PBS; 137 $mmol^{-1}$ NaCl (Sigma, St. Louis MO, USA), 2.7 mmol−1 KCl (Sigma, St. Louis MO, USA), 8.1 $mmol^{-1}$ $Na_2HPO_4 \cdot 2H_2O$, 1.76 $mmol^{-1}$ KH_2PO_4 (Sigma, St. Louis MO, USA), pH 7.4] and to 5 mL of TSA soft were added 500 µL of the phage and 200 µL of *Pseudomonas sp*. suspensions which were placed on a Petri plate containing Tryptic Soy Agar (TSA, Liofilchem, Roseto degli Abruzzi (TE), Italy). Plates were incubated at 25 ˚C for 18 h and formed plaques were expressed as plaque-forming units per milliliter (PFU mL^{-1}). A spot test was also done to confirm the phage stock purity. For that, 5 mL of TSA soft with 200 µL of bacteria was added to a plate with TSA and later 20 µL of the phage stock was added to this plate. The plate was incubated as described above.

2.4. Wastewater sample collection

Composite wastewater samples were collected from a wastewater treatment plant (WWTP) located at the litoral center of Portugal. The WWTP receives wastewater from both domestic and industrial facilities. The WW composite collection was the resultant of a series of individual samples

taken from the secondary treatment station, over a total period of 24 h. The composite samples were collected on different days. After the collection, the WW samples were kept in the dark and refrigerated at 4 ˚C, until further use.

2.5. Assessment of the survival of phage ϕ6 under different environmental conditions

The effects of temperature, pH, salinity, and radiation (UV-B and sunlight) on phage ϕ6 viability (final concentration of 10^7 PFU mL^{-1}) were tested in 10 mL of WW filtered by 0.22 µm pore membranes and sterilized (by autoclave procedure). During the experiments, aliquots of the samples were collected to determine the phage titer. The aliquots were serially-diluted in PBS and plated by the double-layer agar method. Plates were incubated at 25 ˚C for 18 h. Three independent trials were performed for each condition. The end of the experiments was considered when non-detection of viral lysis plaques was achieved.

2.5.1. Temperature experiments

To evaluate the effect of temperature upon phage viability, phage suspension was added to previously prepared WW samples, and the samples were maintained at defined and constant temperatures of 17, 25, and 37 ˚C in an incubating chamber. To assess the effect of the selected temperatures, aliquots were collected every day during the first week (days 0, 1, 2, 3, 4, 5, 6, and 7 of incubation), followed by a once-a-week collection, until the end of the experiments.

2.5.2. pH experiments

In order to evaluate the effect of pH upon phage viability, suspensions of phage ϕ6 were added to previously prepared WW samples with adjusted pH values of 6, 8, and 9. To obtain the desired pH values, acidic/basic solutions (HCl/NaOH) were added to the WW samples as needed. During these experiments, the temperature of the samples was kept at 17 ˚C. To assess the effect of the selected pH, aliquots were collected every day during the first week (days 0, 1, 2, 3, 4, 5, 6, and 7 of incubation), followed by a once-a-week collection, until the end of the experiments.

2.5.3. Salinity experiments

In order to evaluate the effect of salinity upon phage viability, phage suspensions were added to the previously prepared WW samples with salinity values adjusted to 34 g kg^{-1} and 15 g kg^{-1} by adding artificial seawater medium Tropic Marin® Pro-Reef (Tropic Marin®, Wartenberg, Germany). The samples were maintained at 17 ˚C during the experiments. A control sample was made without changing the salinity value and kept at the same conditions. To assess the effect of the selected salinity, aliquots were collected every day during the first week (days 0, 1, 2, 3, 4, 5, 6, and 7 of incubation), followed by a once-a-week collection, until the end of the experiments.

2.5.4. UV-B irradiation experiments

To evaluate the effect of UV-B irradiation (290–320 nm) on phage viability, an ultra-violet type B lamp TL 20 W/12 RS (Philips, Holland) was used and placed at a distance of 25 cm from the samples. The experiments were performed in previously prepared WW samples and PBS, and the temperature was controlled during the experiment and maintained at 17 ˚C. The control samples were incubated in the same conditions as the test samples but were not exposed to UV-B radiation. To assess the effect of UV-B irradiation, aliquots were collected after 0, 2, 4, 6, 8, 10, and 12 h of exposure, until the end of the experiments.

2.5.5. Solar radiation experiments

To evaluate the effect of solar radiation upon phage viability, phage ϕ6 suspensions were added to previously prepared WW samples and exposed to natural solar radiation. The control samples were incubated in the same conditions as the test samples but were not exposed to solar radiation. The experiments were performed under a solar irradiance from 46.2 to 91.1 mW cm^{-2} in a day with an ambient temperature ranging from 13.5 to 16.7 ˚C. During the experiments, aliquots were collected after 0, 2, 4, and 6 h of exposure to natural solar irradiation.

2.6. Antimicrobial photodynamic therapy treatments

The used photosensitizer 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (TetraPy(+)Me) was prepared according to the literature (Carvalho et al., 2010). Its 1H NMR and UV–vis spectra were consistent with the literature data. The purity was confirmed by thin-layer chromatography and 1H NMR. 1H NMR (DMSO-d 6): −3.12 (s, 2H, NH), 4.73 (s, 12H, CH_3), 9.00 (d, J = 6.5 Hz, 8H, Py-o-H), 9.22 (s, 8H, β-H), 9.49 (d, J = 6.5 Hz, 8H, Py-m-H). UV–vis (DMSO) λmax (log ε): 425 (5.43), 516 (4.29), 549 (3.77), 588 (3.84), 642 (3.30) nm. The stock solution (500 μM) of this porphyrin was prepared using the polar aprotic solvent dimethyl sulfoxide (DMSO) and stored at room temperature, protected from light.

The assays were performed in PBS and in 0.22 μm filtered WW in 6-well plates with a final volume of suspension of 5 mL per sample. To the PBS or filtered WW, a determined phage stock volume was added to each sample well to achieve the initial phage concentration of ca. 10^7 - 10^8

PFU mL^{-1}. The amount of PS TetraPy(+)Me was added to reach a final concentration of 5.0 µM. Light and dark controls were performed alongside the PDI samples: in the light controls (LC), a phage suspension in PBS or filtered WW was exposed to light without PS addition; in the dark controls, the phage suspension in PBS or filtered WW, containing the PS at the same concentration as in the samples, was protected from light wrapped in aluminum foil during the PDI treatment. Before the irradiation procedure (pre-irradiation period), samples and controls were incubated for 10 min with shaking at room temperature to promote the binding of porphyrin to phage ϕ6. Then, the samples and LC were exposed to a white light-emitting diode (LED) system (EL®MARK, 20 W, ~230 V, and ~50 Hz) with an irradiance of 50 mW cm^{-2}, adjusted with a power meter FieldMaxII-Top (Coherent, Santa Clara, CA, USA) connected to a high sensitivity sensor PS19Q (Coherent, Santa Clara, CA, USA). During the experiments, the samples were under magnetic stirring, and aliquots of 100 µL of samples and controls were taken at times 0, 5, 10, 15, and 30 min, serially diluted in PBS and plated with the host, by the drop plated method in Petri dishes previously prepared with TSA and a layer of TSA-soft with the phage host *Pseudomonas sp*. for monitoring the phage survival. Three independent assays were performed for each condition.

2.7. Effect of the PDI treated effluent on native marine water microorganisms

A sample (ca. 5 L) of coastal marine water was collected in the litoral center of Portugal. This sample was filtered with a 1.2 µm pore-size membrane to remove the suspended matter, followed by a second filtration with a 0.22 µm pore-size membrane to remove the remaining native bacteria. The two-times filtered marine water sample was sterilized by moist heat to ensure the inactivation of residual microorganisms (as viruses) whose dimensions did not allow its retention by the used membranes. The sterilized marine water samples were stored, protected from light, at 4 ˚C, until further use.

On the day of the assay, new coastal marine water samples were collected. The number of total cultivable microorganisms was determined using Plate Count Agar (PCA, Liofilchem, Roseto degli Abruzzi (TE), Italy) culture medium: a volume of 1.0 mL of the collected marine water was plated by incorporation in PCA medium in Petri dishes; the plates were incubated during 18 h, at 25 ˚C; after incubation, the content of the plate was counted and the results expressed in CFU mL^{-1}. The collected water marine samples were pre-filtered by a 1.2 µm to remove the suspended matter. After the pre-filtration step, a volume of 500 mL of the pre-filtered samples was filtered by a 0.22 µm pore-size mem-brane, and the retained content of the filter was resuspended in 5.0 mL of the sterilized marine water previously prepared, to concentrate the native marine water microorganisms, hereinafter referred to as “native marine microorganisms concentrate”, for ease of identification called. The number of total cultivable microorganisms was quantified again, as previously described, in PCA medium, and the prepared suspension was stored until further use.

PDI experiments were carried out under the same conditions mentioned in section 2.6 (TetraPy(+)Me as the used PS, at a concentration of 5.0 µM, under a white light irradiance of 50 mW cm^{-2}, for 30 min), but without the addition of any biological entity. To the previously prepared

suspension of resuspended native microorganisms added to sterile marine water, a determined volume of PDI-treated WW was added, and the following samples were performed:

(i) non-irradiated control of the native marine microorganisms concentrate;

(ii) irradiated (50 mW cm-^{2}) control of the native marine microorganisms concentrate;

(iii) non-irradiated controls of filtered WW added to the native marine microorganisms concentrate in the ratios of 1:2, 1:10, 1:100, 1:1000 (WW: native marine microorganisms concentrate);

(iv) irradiated (50 mW cm^{-2}) controls of filtered WW added to the native marine microorganisms concentrate in the ratios of 1:2, 1:10, 1:100, 1:1000 (WW: native marine microorganisms concentrate);

(v) non-irradiated samples, with PDI-treated filtered WW added to native marine microorganisms concentrate in the ratios of 1:2, 1:10, 1:100, 1:1000 (WW: native marine microorganisms concentrate);

(vi) and irradiated samples (50 mW cm^{-2}), with PDI-treated filtered WW added to native marine microorganisms concentrate in the ratios of 1:2, 1:10, 1:100, 1:1000 (WW: native marine microorganisms concentrate).

The replicates were made for a total volume of 10 mL, and the assays were carried out at a constant temperature of 17 ˚C. The irradiation period of the samples and controls lasted 24 h and aliquots of the samples and controls were collected at 0, 6, and 24 h.

From each treated and control sample, tenfold serial dilutions were prepared in sterile PBS (10^{0} to 10^{-6}). Aliquots of 100 µL were pour-plated in PCA. The plates were incubated at 25 ˚C for 18 h and the number of colony-forming units was counted. Three independent assays were performed.

2.7.1. Enumeration of viable cells

From each treated and control sample tenfold serial dilutions were prepared in sterile PBS (10^{0} to 10^{-6}). Aliquots of 100 µL were pour-plated in PCA. The plates were incubated at 25 ˚C for 18 h and the number of colonies was counted. Two independent assays were performed.

2.8. Statistical Analyses

The statistical analysis was done with GraphPad Prism. Normal distributions were checked by Kolmorov-Smirnov test and homogeneity of variances by Brown-Forsythe test. Differences corresponding to $p < 0.05$ were considered significant. ANOVA and Tukey's multiple comparisons test was applied to assess the significance of the differences between the tested conditions. The statistical analysis was performed considering the three independent assays performed for each condition.

3. Results

3.1. Assessment of the effect of environmental factors upon phage ϕ6 viability

3.1.1. Temperature experiments

The decrease in the phage ϕ6 viability varied with the temperature. The decrease was faster at 37 ˚C than at 25 ˚C, which in turn was also faster than at 17 ˚C. At 37 ˚C, after 24 h of the viability of the phage ϕ6 decreased to the detection limit of the method, but at 12 h the decrease of phage was already 6 log PFU mL^{-1} (Figure 1a). In assays performed at 25 ˚C, the decrease to the detection limit of the method was only observed after 35 days (Figure 1b). At 17 ˚C, the decrease of the phage to the detection limit of the method was only observed after 84 days (Figure 1c).

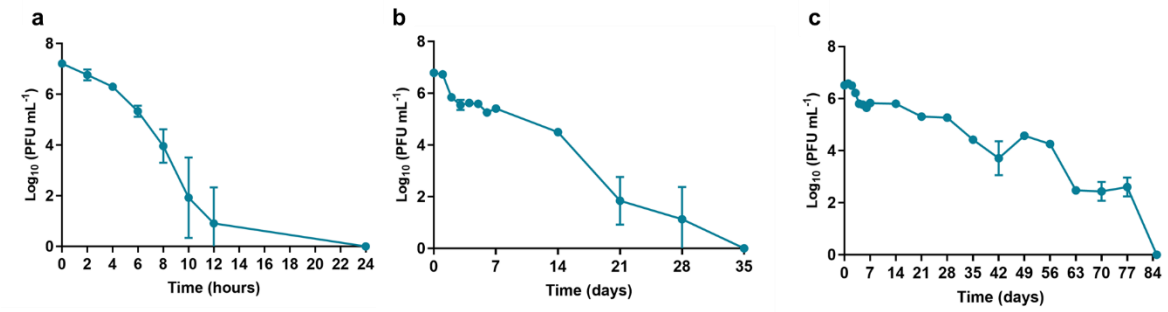

Figure 1: Survival of phage ϕ6 following exposure to different temperature value: 37 ºC (a); 25 ºC (b); 17 ºC (c). Values represent the mean of three independent experiments; error bars represent the standard deviation.

3.1.2. pH experiments

When phage ϕ6 was submitted to different pH values (6, 8, and 9), it was observed that the phage viability behaved similarly in solutions at pH 8 and 9, where a decrease of 7.5 log PFU mL^{-1} was observed after 63 days (Figure 2). In the case of the pH 6, a decrease of 5.7 log PFU mL^{-1} in the phage viability was only observed after 84 days (Figure 2).

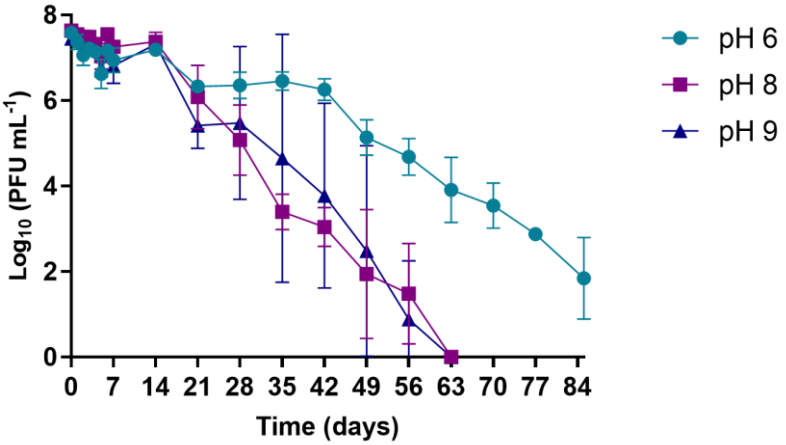

Figure 2: Survival of phage ϕ6 following exposure to different pH value. Values represent the mean of three independent experiments; error bars represent the standard deviation.

3.1.3. Salinity experiments

In the case of the assays where the viability of phage ϕ6 was studied at different salinity conditions, it was observed a decrease in the phage viability of 7.3 log PFU mL^{-1} after 49 days for salinity 34 g kg^{-1}. For the control and for the solution adjusted to 15 g kg^{-1} the phage viability decrease was 6.4 and 5.7 log PFU ml^{-1}, respectively, only after 84 days.

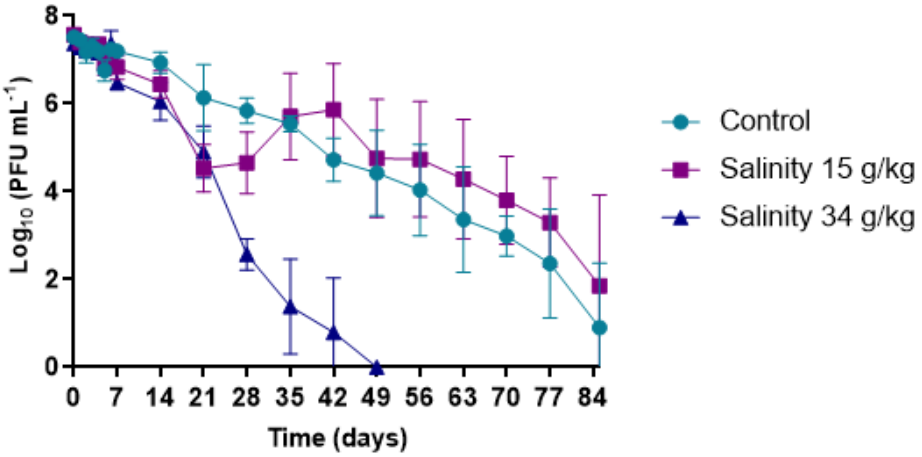

Figure 3: Survival of phage ϕ6 following exposure to different salinity value. Values represent the mean of three independent experiments; error bars represent the standard deviation; phage controls had no change in salinity.

3.1.4. UV-B irradiation experiments

The viability of phage ϕ6, when exposed to UV-B irradiation, decreased during the 12 h of incubation, in both PBS and WW assays, when compared to controls. A maximum decrease of approximately 7.2 and 7.5 log PFU mL^{-1} was observed after 12 h in the WW and PBS assays, respectively (Figure 4). The concentration of phage ϕ6 not exposed to UV-B radiation remained constant in both PBS and WW assays during the 12 h of incubation (Figure 4).

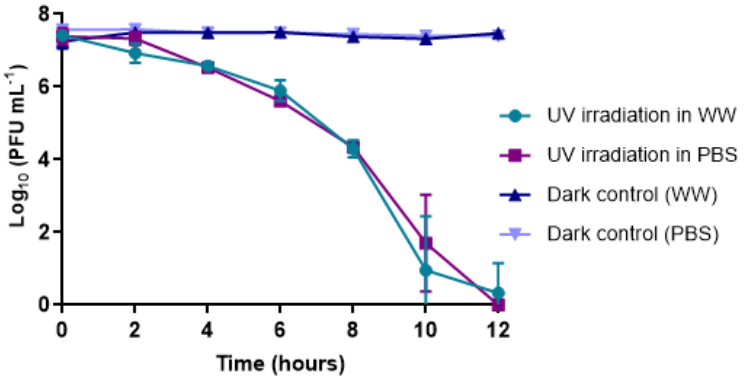

Figure 4: Survival of phage ϕ6 following exposure to UV-B radiation. Assays were performed in PBS and WW. Values represent the mean of three independent experiments; error bars represent the standard deviation; phage controls were not exposed to UV-B radiation.

3.1.5. Solar radiation experiments

When phage ϕ6 in PBS was exposed to solar radiation, the abundance of phage decreased by 2.6 log PFU mL^{-1} (Figure 5) at the end of the 6 hours, when compared to the phage control. A higher difference was observed when the phage ϕ6 in WW was exposed to solar radiation, a decrease of 7.5 log PFU mL^{-1}at the end of 4 hours was observed (Figure 5), when compared to the respective control.

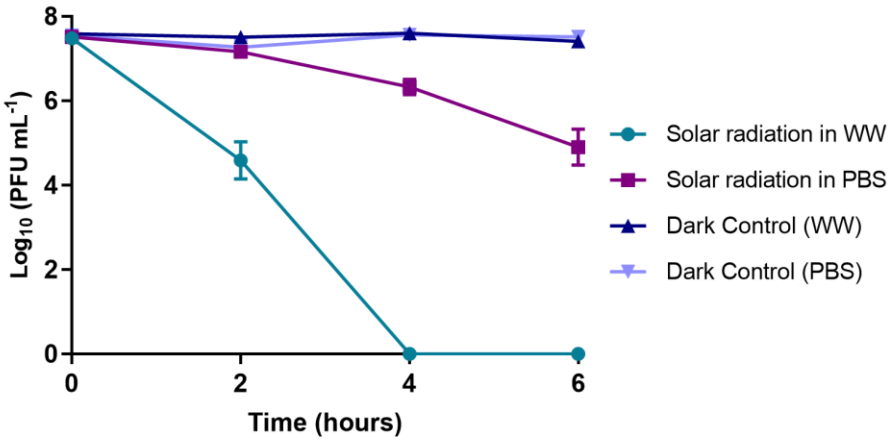

Figure 5: Survival of phage ϕ6 following exposure to solar radiation. Assays were performed in PBS and WW. Values represent the mean of three independent experiments; error bars represent the standard deviation; phage controls were not exposed to solar radiation.

3.2. Photodynamic inactivation of phage ϕ6 by PDI

The results of phage suspensions in PBS exposed to 60 m of PDI treatment (TetraPy(+)Me at 5.0 µM, and irradiance of 50 mW cm^{-2} showed that phage ϕ6 was efficiently inactivated by PDI after 5 min of treatment (by more than 3 log), reaching the detection limit of the method after 10 min of treatment (reduction of 8 log), (Figure 6).

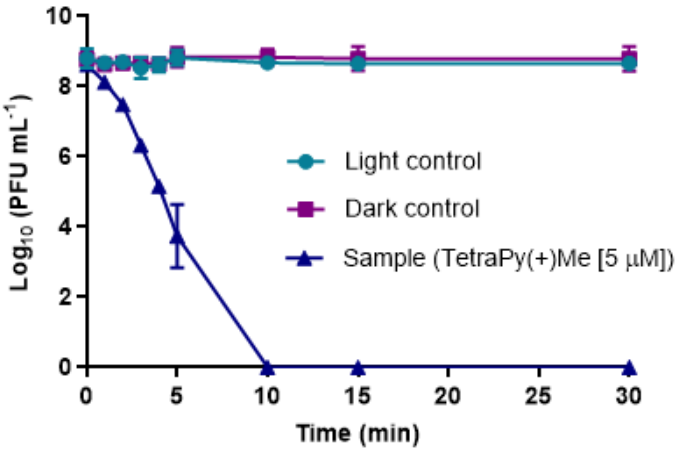

Figure 6: PDI using tetracationic porphyrin TetraPy(+)Me and with light (LED) at 50 mW cm^{-2}, in the inactivation of bacteriophage ϕ6, in PBS. Values represent the mean of three independent experiments; error bars represent the standard deviation; phage controls were not exposed to solar radiation.

PDI assays were also carried out in WW under the same conditions as the PBS treatments. These assays were carried out with WW collected on three different days (October, December 16th and December 18th - 2020) and three independent tests were carried out for each water sample. The phage ϕ6 were efficiently inactivated (reduction of 8 log) by PDI after 30 minutes of treatment in all assays (Figure 7). In the studies carried out in the WW collected in October, the phage was inactivated after 10 minutes of treatment (Figure 7a) to the detection limit of the method. In the case of water collected on December 16, the photoinactivation to the detection limit of the method occurred after 30 minutes of irradiation, however at 5 minutes there was already a phage decrease of 7.7 log PFU mL^{-1} (Figure 7b). In the case of WW collected on December 18, the photoinactivation to the detection limit of the method was observed after 5 minutes of treatment (Figure 7c).

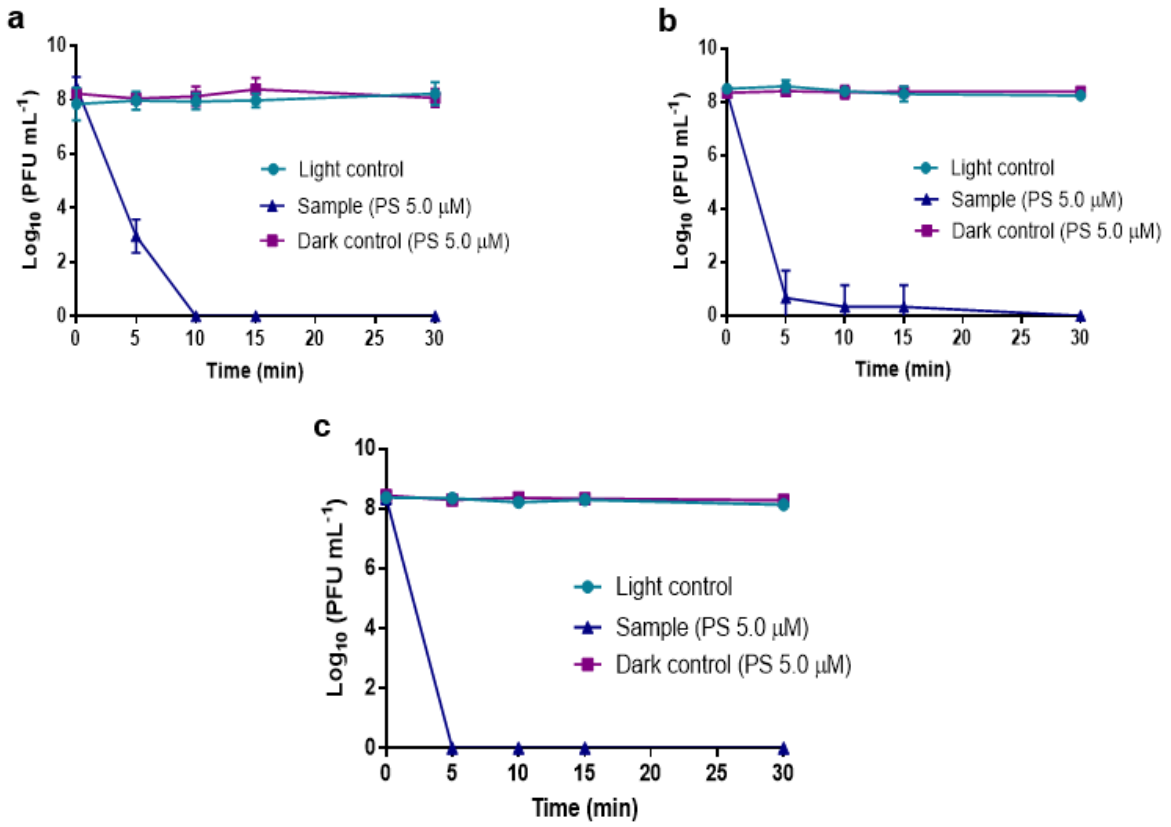

Figure 7: PDI using tetracationic porphyrin TetraPy(+)Me and with light (LED) at 50 mW cm^{-2}, in the inactivation of bacteriophage ϕ6, in WW collected on three different days: October (a); December 16th (b); December 18th (c). Values represent the mean of three independent experiments; error bars represent the standard deviation; phage controls were not exposed to radiation.

3.3. Effect of the PDI treated effluent on native marine water microorganisms with PDI

On the day of the assay, new coastal marine water samples were collected and the total cultivable native marine microorganisms were quantified, and the value of 2.8 log CFU mL^{-1} was obtained. After the concentration process, the total cultivable native marine microorganisms were quantified again, revealing the increase of the total cultivable microorganisms to 3.9 log CFU mL^{-1} (Figure 8).

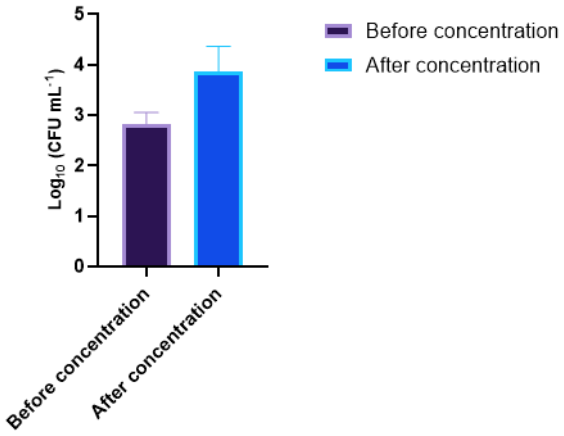

Figure 8: Quantification of the total cultivable native marine microorganisms before and after the concentration process.

In these experiments, only the native marine bacteria of the light sample at a concentration of 1:2 were affected (Figure 9c). The bacterial concentration of the other samples was constant throughout the 24 h of the experiment (Figure 9a, b, and d).

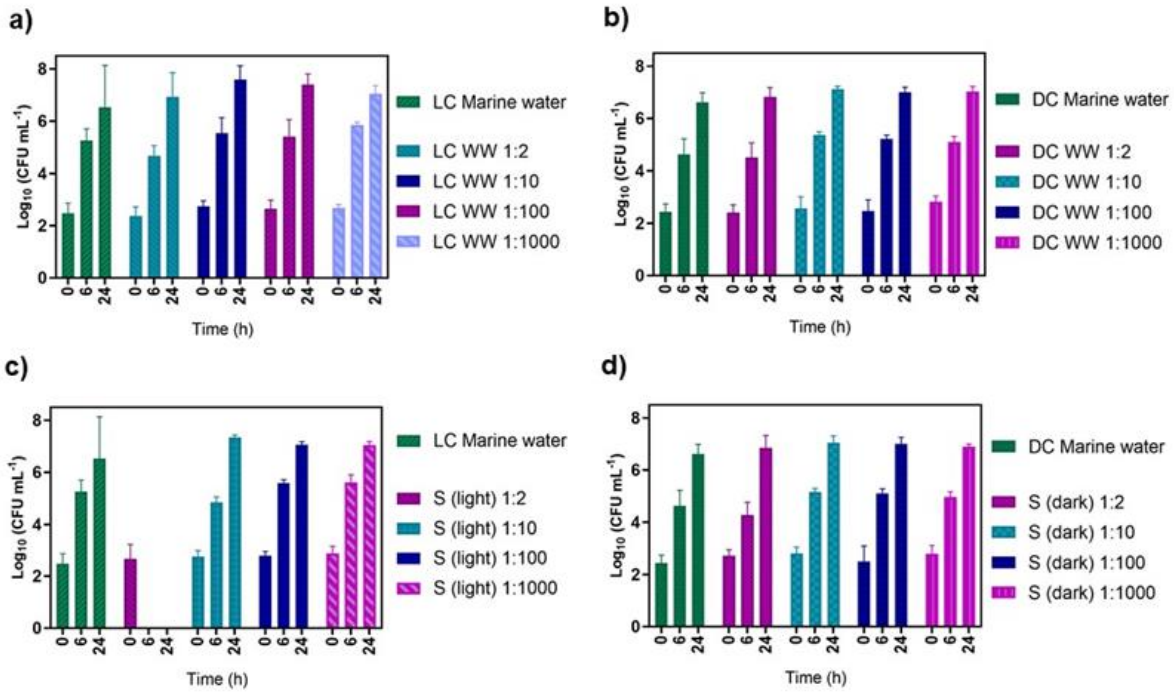

Figure 9: Survival of native marine water bacteria after exposure to light in WW treated previously with PDI: (a) light controls: LC Marine Water: marine water light control; LC WW: WW light control (at different concentrations); (b) dark controls: DC Marine Water: marine water dark control; DC WW: WW dark control (at different concentrations); (c) samples exposed to the light: S (light): light sample

(at different concentrations); (d) samples kept in the dark: S (dark): samples protected from light (at different concentrations). Values represent the mean of two independent experiments.

4. Discussion

Since the main objective of this work was to evaluate the potential application of PDI for the inactivation of the phage ϕ6 (as a predictive model of the mammalian virus SARS-CoV-2) in WW, it was crucial to know first the viability of the virus in different environmental conditions, namely temperature, pH, salinity, solar and ultraviolet radiation.

Temperature is of great importance in the viability of the viruses (Nasser & Oman, 1999; Olson et al., 2004). In this study, the phage viability was tested in WW at 37, 25, and 17 ˚C, with 17 ˚C being the closest temperature to the annual average temperature of seawater in central coastal Portugal (*Portugal: Temperatura Da Água Do Mar*, n.d.), where WW is released after treatment, and also in the WWTPs. The data obtained (Figure 1) in this study corroborate the data previously obtained in PBS by Pinheiro et al (Pinheiro et al., 2019), showing that the viruses less viable at higher temperatures (37 ˚C) than at lower temperatures (17 ˚C), which remains active for up to 84 days of incubation. Since 17 ˚C is the closest temperature of the environment where the wastewater will be released and corresponds to the temperature at which the phage remained viable for the longest time and also to the temperature closer to that of the WWTPs, the results obtained confirm the need for an effective way of inactivating the phage from WW.

pH is another important factor influencing phage stability in the environment (Jończyk et al., 2011). The pH values tested in this study (6, 8, and 9) correspond to the range of WW emission values, within this range the pH value of marine waters is also included. The data obtained (Figure 2) show that the virus remains viable on WW over several days (63 days for pH 8 and pH 9 and, at least, 84 days for pH 6) for all pH values, which reiterates the need for WW viral inactivation before discharge in the environment.

Considering that marine water is one of the places where WW is released after treatment, salinity is also an important factor to take into account. As such, the phage was incubated in WW with salinity values of 15 g kg^{-1} and 34 g kg^{-1} (corresponding, respectively, to the values of brackish and marine water). In these assays it was found that the phage viability decreases in more saline environments, remaining, however, viable for 49 days (Figure 3).

One of the factors recognized as the most important for the loss of virus infectivity in the environment is solar radiation, or more specifically, UV radiation (Duarte et al., 2018; C. David Lytle & Sagripanti, 2005; Mojica & Brussaard, 2014; Silva et al., 2014; Wommack et al., 1996). Solar radiation can directly affect free viruses, degrading proteins, altering the structure, and decreasing infectivity (Mojica & Brussaard, 2014). UV-B radiation, on the other hand, causes irreversible damage to the genomic material and can lead to the modification of viral proteins and the formation of (lethal) photoproducts (Mojica & Brussaard, 2014; Rule Wigginton et al., 2010). Effectively, as in the study of Pinheiro et al. (Pinheiro et al., 2019), a decrease in the abundance of phage ϕ6 particles, both in

PBS and WW, was observed when exposed to solar radiation and UV-B radiation, without viable viral particles after 4 and 12 hours, respectively (Figures 4 and 5).

Since phage ϕ6 is composed of RNA, proteins, and phospholipids (Laurinavičius et al., 2004), and these molecules are potential targets for viral photoinactivation (Wiehe et al., 2019), it is expected that the virus is sensitive to light, even though there is no direct evidence of the existence of endogenous PS (Vatter et al., 2020). According to the literature, phage ϕ6 has already proved to be sensitive to violet irradiation from 405 nm (Vatter et al., 2020), and 455 nm wavelength visible light (Vatter et al., 2021), without any added PS. Although the phage does not contain endogenous PS, it is speculated that the virus may carry bacterial PS from its host (*P. syringae*) when it assembles its envelope (Vatter et al., 2021).

It has also been shown by Tomb et al (Tomb et al., 2014, 2017), that phage ϕ6 is inactivated by external PS, namely by porphyrins, during illumination with visible light. This effect is explained by the production of ROS (formed during the irradiation process), which attack the lipids of the envelope, the proteins of the capsid, and the nucleic acids of the viruses (Costa et al., 2012).

Photodynamic treatment has been the subject of many, *in vitro* and *ex vivo*, studies and is already used in the inactivation of other viruses, namely in the treatment of the Herpes Simplex Virus (HSV) (Marotti et al., 2010; Nobbe et al., 2011; Osiecka et al., 2017; Zverev et al., 2016), of Papilloma Human (HPV) (Caucanas et al., 2010; Q. Li et al., 2014; Y. G. Lu et al., 2010; L. Wu et al., 2019) and also Varicella-Zoster Virus (VZV) (Teitelbaum et al., 2020). The efficiency of PDI has also been studied for the treatment of bacterial lung infections (Biel et al., 2012; Geralde et al., 2017; Kassab et al., 2020).

As such, PDI was already considered in the theoretical treatment of COVID-19, as it triggers viral inactivation, especially of enveloped viruses such as SARS-CoV-2 (Willis et al., 2021) in the clinical field.

Regarding WW, and although there are still no experimental studies in the literature on the ability of PDI to inactivate SARS-CoV-2 in WW, it is expected that PDI can be an efficient approach to disinfect WW. In addition to the fact that PDI has already demonstrated its ability to inactivate other viruses, namely enveloped viruses such as CoVs (Costa et al., 2011, 2012), different PSs have already been shown to be effective in the photodynamic inactivation of viruses in secondarily treated WW (Alves et al., 2008; Carvalho et al., 2007). The results obtained throughout this work support the theory that PDI can be an efficient alternative for the inactivation of SARS-CoV-2 in secondarily treated WW, as a tertiary treatment approach to WW disinfection, once very promising results were obtained: a viral load (phage ϕ6 used as a SARS-CoV-2 surrogate) > 8 log PFU mL^{-1} was photoinactivated after just 10 minutes of treatment using the porphyrinic derivative TetraPy(+)Me on the micromolar scale (5.0 μM) in real WW (Figure 7). However, although the phage ϕ6 has been considered a suitable model of the SARS-CoV-2, further studies using the SARS-CoV-2 are needed. In order to develop a safe WW treatment protocol, it is crucial to understand if the PDI would affect the native marine aquatic microorganisms, where the treated effluents are discharged. The results of this study showed that only in the case of the less diluted light samples (1:2) the viability of the

native bacteria was negatively affected. These results are expected, since the PS is still present in the solution in the less diluted light samples, reacting with light and producing ROS. However, at higher dilutions, this effect is no longer significant as the concentration of PS present in the samples is lower. As the WW when is discharged in the marine environment is greatly diluted, the potential impact on the aquatic organisms should not be high. In a study conducted by Ramos and Neves (Ramos & Valente Neves, 2009) the authors monitored the discharge of wastewater occurring through an underwater sewage outlet (in the central coastal region of Portugal). With the data collected with the help of a autonomous underwater vehicle, the authors presented predictive mathematical models, based on the theory of jets (effluent behaviour close to the source of flow) and plumes (resulting from the behaviour of the effluent far from the source of flow), and were able to map the dispersion of the plume and its dilution, as a function of physico-chemical parameters such as the temperature of the effluent and the receiving waters, and also of salinity differences. Additionally, ocean currents also play an important role in the dispersion/dilution of the effluent in the receiving waters. At the given submarine exutor, the average discharge flow rate is about 0.8 m^3/s and occurs at about 15 m depth. The dilution estimates are consistently greater than 30 (in the initial mixing zone, between 15 and 11 m depth), with plume dilution being estimated at more than 300 up to 8 m depth.

Nevertheless, more studies are needed in order to evaluate the probable impact that the PDI treatment could have if applied in a real context, in a WWTP.

5. Conclusions

In general, it can be concluded that (i) the phage ϕ6 (used as SARS-CoV-2 surrogate) remains viable in the environment for a considerable time under conditions similar to those of the environment in which WW are released after treatment and of the WWTPs where the WW is treated; (ii) the PDI process is effective to inactivate the phage ϕ6 in WW; (iii) and the effluent, at higher dilution rates in seawater, does not produce toxicity to the native marine aquatic microorganisms.

It is important to note that the results were obtained for phage ϕ6, which is considered a suitable model of SARS-CoV-2, but further studies using the SARS-CoV-2 are necessary.

6. References

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