Sewage bacteriophage inactivation by cationic porphyrins: influence of light parameters

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Photodynamic therapy has been used to inactivate microorganisms through the use of targeted photosensitizers. Although the photoinactivation of microorganisms has already been studied under different conditions, a systematic evaluation of irradiation characteristics is still limited. The goal of this study was to test how the light dose, fluence rate and irradiation source affect the viral photoinactivation of a T4-like sewage bacteriophage. The experiments were carried out using white PAR light delivered by fluorescent PAR lamps (40 W m^{-2}), sun light (600 W m^{-2}) and an halogen lamp (40–1690 W m^{-2}). Phage suspensions and two cationic photosensitizers (Tetra-Py^+-Me, Tri-Py^+-Me-PF) at concentrations of 0.5, 1.0 and 5.0 μM were used. The results showed that the efficacy of the bacteriophage photoinactivation is correlated not only with the sensitizer and its concentration but also with the light source, energy dose and fluence rate applied. Both photosensitizers at 5.0 μM were able to inactivate the T4-like phage to the limit of detection for each light source and fluence rate. However, depending of the light parameters, different irradiation times are required. The efficiency of photoinactivation is dependent on the spectral emission distribution of the light sources used. Considering the same light source and a fixed light dose applied at different fluence rates, phage inactivation was significantly higher when low fluence rates were used. In this way, the light source, fluence rate and total light dose play an important role in the effectiveness of the antimicrobial photodynamic therapy and should always be considered when establishing an optimal antimicrobial protocol.

Introduction

As human population densities increase, it becomes more and more difficult to provide supplies of high-quality potable water from surface and ground water stocks, and the removal of harmful microorganisms, such as bacteria, viruses and protozoa, assumes greater significance. Although the transmission of microbial diseases 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. Currently, photodynamic therapy is receiving considerable interest as a potential antimicrobial treatment. Photosensitizers, 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.[1–3]

Photodynamic antimicrobial phototherapy (PACT) has been proven to be a powerful method for inactivating viruses, such as murine retroviral vectors, human immunodeficiency viruses (HIV-1 and -2),[4,5] hepatitis viruses (A and B),[6,7] vesicular stomatitis virus (VSV),[8] herpes simplex viruses,[9–11] human papillomavirus[12] and influenza A.[13] The effect of PACT on bacterial viruses (bacteriophages or simply phages), frequently used as indicators of enteric viruses and public health risk, has already been tested with success not only on collection phages[14–19] but also on sewage bacteriophages.[3,20,21]

Several studies indicate that the physicochemical properties of the sensitizer, namely the light-absorption characteristics and the efficiency of singlet oxygen and free radicals production, have an impact on the efficacy of photosensitization.[22–24] Photosensitization occurs when the photosensitizer (PS) is able to absorb and transfer the energy of the incident light to molecular oxygen leading to the formation of cytotoxic species. However, it is recognized that in addition to the PS properties, the characteristics of the incident light must be considered and controlled to increase the efficiency of PACT.[25] In the literature, light parameters are usually discussed in terms of light dose (also identified as fluence)[1,21,25–27] but, as the restriction of the reciprocity rule to rather narrow limits for most photobiological reactions, the dose–time relationship (fluence rate) should also be taken into account.[28] A very wide selection of light sources are available, ranging from thermal radiators, which are heated until they start glowing and thus emit light (for instance, incandescent lamps) to luminescent radiators, which utilize the electronic excitation of atoms to emit light (for instance, gas discharge lamps, light emitting diodes (LED) and lasers).[24,29]

Recently we have shown that porphyrins with three or four positive charges are able to inactivate environmental non-enveloped viruses, allowing them to be used as a new, cheap and accessible technology for wastewater treatment.[21] In order to establish the best conditions for an efficient photoinactivation of somatic bacteriophages, we have studied how phage

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inactivation, in the presence of efficient PS, is affected by: (i) light source, (ii) light dose and (iii) fluence rate. For this purpose, we selected the T4-like sewage bacteriophage, used as indicator of the presence of enteric viruses, and the photosensitizers 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py$^+$-Me) and 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py$^+$-Me-PF). Tri-Py$^+$-Me-PF is a tricationic porphyrin recently described by our group as a promising PS for the inactivation of several types of microorganisms and Tetra-Py$^+$-Me is the most extensively studied PS in bacterial and viral photoinactivation processes.[3,21,30]

Results

The photocytotoxicity action on the T4-like sewage bacteriophage by the two cationic porphyrin derivatives (Fig. 1), was initially assessed by exposing the test assembly to different photosensitizer concentrations (0.5, 1.0 and 5.0 μM) and to white light from different light sources and fluence rates.

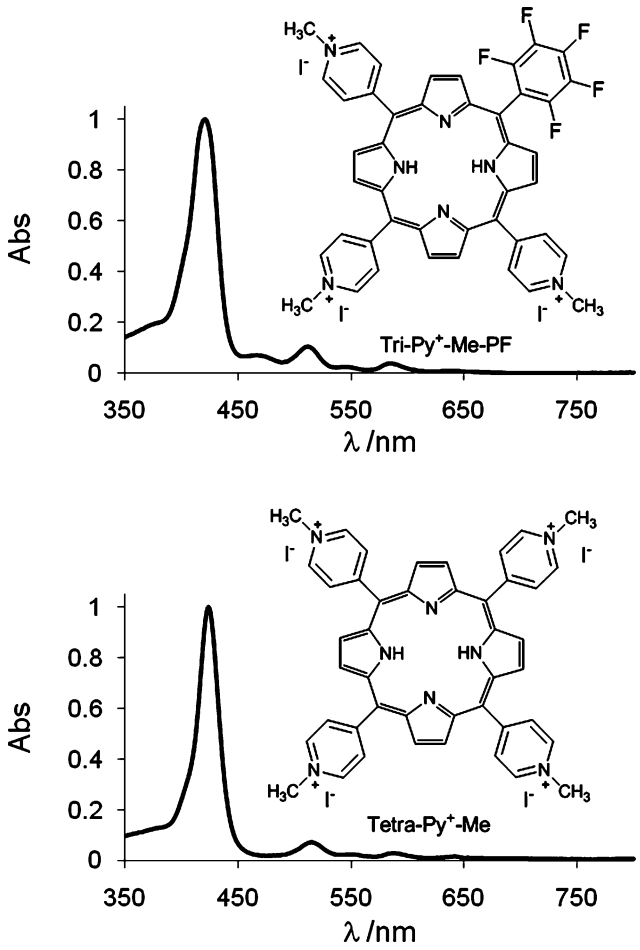

Fig. 1 Structure and UV-Vis spectra (in DMSO) of the two cationic porphyrins used in this study for the photoinactivation of T4-like bacteriophage.

Bacteriophage host viability

The bacteriophage host viability test showed that the pattern of phage inactivation was not affected by the presence of photosensitizer during the incubation period (18 h) in the dark. The pattern of phage inactivation was similar in washed and in non-washed samples.[21] This means that the photosensitizer does not affect the bacteria during incubation of the Petri plates in the dark and, therefore, the washing step by ultra-centrifugation, a time consuming procedure, was not carried out. However, in all the experiments, the Petri plates were kept in the dark during the incubation period.

Bacteriophage viability

Without light (dark control), the photosensitizers at the highest concentration (5.0 μM) did not exhibit activity against the phage for the exposure time (270 min). A similar trend was observed for the phage in the absence of the PS for all the irradiation periods with white light (light control) (Fig. 2 to 6). It is important to note that the light used in the experiments did not affect viral viability. Inactivation was observed only when the phage was incubated with the photosensitizer and irradiated with the appropriate light dose.

Influence of light sources on bacteriophage inactivation

The efficiency of the sewage bacteriophage inactivation for the two cationic porphyrin derivatives irradiated with selected light sources was different. Although the two cationic porphyrins at 5.0 μM when activated by all the light sources were able to inactivate the sewage T4-like phage to the limit of detection, the phage photoinactivation with a halogen lamp occurred faster than with solar irradiation or with fluorescent lamps (Fig. 2 to 4).

In fact, when the illumination system (halogen lamp) was used at a fluence rate of 1690 W m^{-2}, the Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me porphyrins, at a concentration of 5.0 μM, were able to inactivate the sewage T4-like phage by more than 7 log (Fig. 2) after 25 and 45 min (data not shown), respectively. Under these light conditions, for a concentration of 1.0 μM, both photosensitizers produced a moderate phage inactivation after 30 min of exposure to light (2.2 and 1.3 log for Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me). For the lowest concentration of photosensitizers (0.5 μM) the inactivation varied between reductions of 0.8 to 1.3 log after 30 min of irradiation (Fig. 2). With these light conditions (1690 W m^{-2}) the pattern of phage inactivation was similar for both porphyrins (ANOVA, $p > 0.05$), for all the concentration tested (Fig. 2).

Under solar light (~600 W m^{-2}), the T4-like phage was also efficiently photoinactivated (>99.9999% of inactivation) with reductions of 7.2 log for Tri-Py$^+$-Me-PF (5.0 μM) after 90 min and 7.0 log for Tetra-Py$^+$-Me (5.0 μM) after 180 min of irradiation (data not shown). With 1.0 μM of sensitizer, the inactivation ranged from 3.1 log to 0.8 log after 90 min of exposure for Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me, respectively. For concentrations of 0.5 μM, the reductions observed were 1.5 log 0.1 log (after 90 min), for Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me, respectively (Fig. 3). With this light source, the pattern of phage inactivation was similar for Tri-Py$^+$-Me-PF and Tetra-Py$^+$-Me, but only for the highest concentration (5.0 μM) (ANOVA, $p > 0.05$). For the other concentrations (0.5 and 1.0 μM) the pattern of phage inactivation was different for both porphyrins (ANOVA, $p < 0.05$) (Fig. 3).

Porphyrins irradiated with a set of 13 fluorescent lamps (40 W m^{-2}), at 5.0 μM, also inactivated the T4-like phage to the

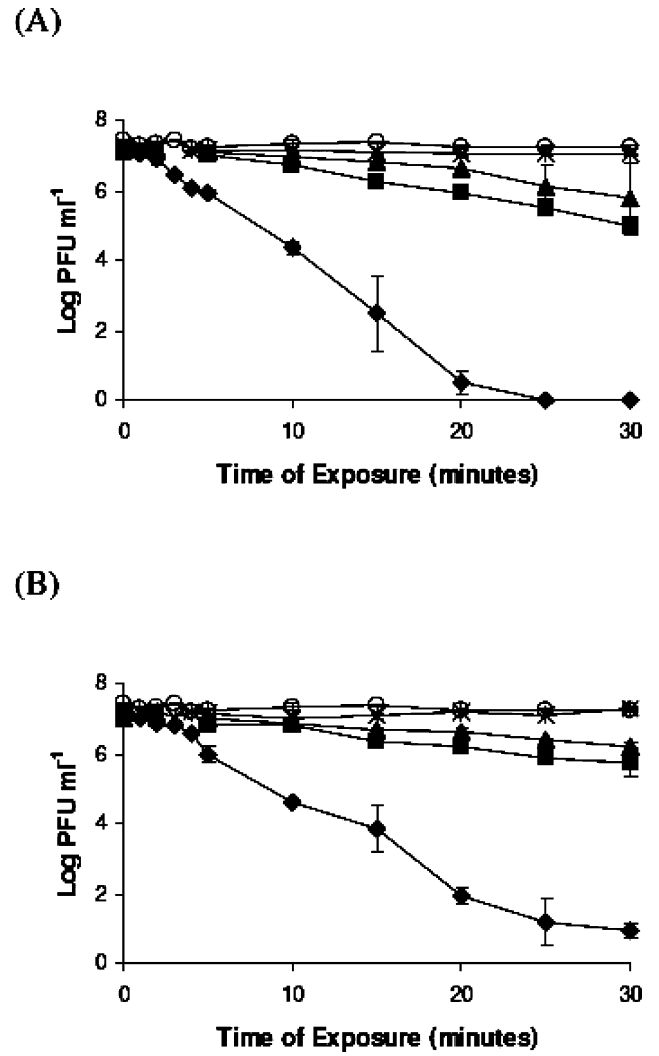

Fig. 2 Density variation of the sewage bacteriophage after irradiation with the illumination system (halogen lamp) at 1690 W m^{-2} in the presence of Tri-Py^+-Me-PF (A) and Tetra-Py^+-Me (B), respectively. (○ light control, * dark control, ▲ 0.5 μM, ■ 1.0 μM, ♦ 5.0 μM). Error bars represent standard deviations.

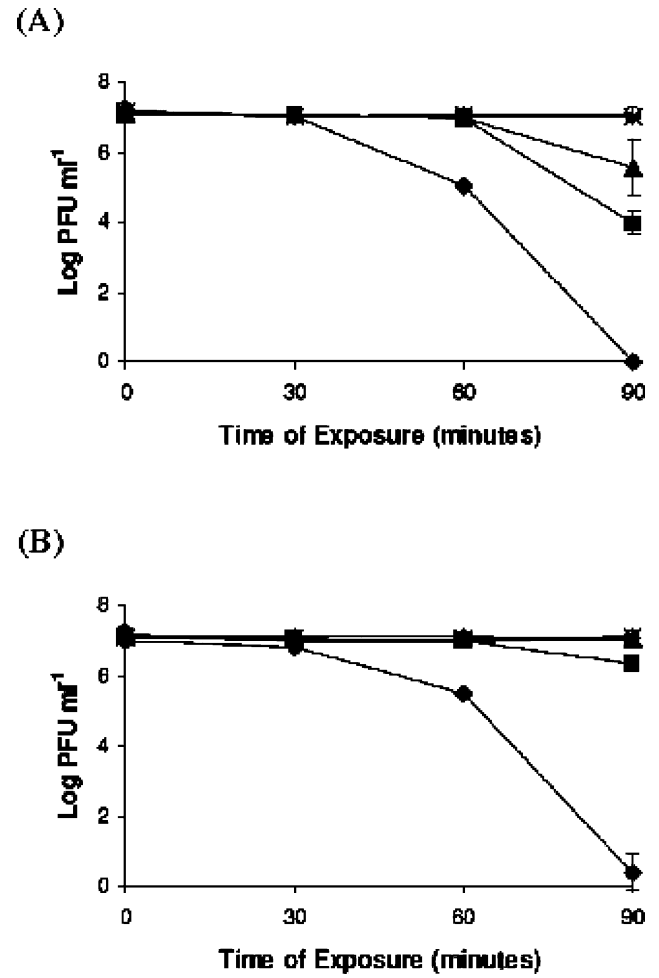

Fig. 3 Density variation of the sewage bacteriophage after irradiation with solar light (600 W m^{-2}) in the presence of Tri-Py^+-Me-PF (A) and Tetra-Py^+-Me (B), respectively. (○ light control, * dark control, ▲ 0.5 μM, ■ 1.0 μM, ♦ 5.0 μM). Error bars represent standard deviations.

limit of detection with reductions of 7.0 log (after 180 min of irradiation) and 7.2 log (after 270 min of irradiation) for Tri-Py^+-Me-PF and Tetra-Py^+-Me, respectively. Again, for the lower concentrations (0.5 and 1.0 μM) the inactivation was moderate and ranged from reductions of 3.6 log and 1.5 log for 1.0 μM and reductions of 1.8 to 0.9 log for 0.5 μM, respectively, for Tri-Py^+-Me-PF and Tetra-Py^+-Me, after 270 min of exposure to white light (Fig. 4). Also, for both porphyrins, with this light source the pattern of phage inactivation was similar for the highest concentration (5.0 μM) (ANOVA, $p > 0.05$) and different for the other concentrations (0.5 and 1.0 μM) (ANOVA, $p < 0.05$) (Fig. 4).

The results of the comparison of the efficacy of sewage T4-like bacteriophage inactivation by Tri-Py^+-Me-PF using the same fluence rate delivered by different light sources are presented in Fig. 5 and 6. The results show that the efficacy of phage inactivation at a low fluence rate (40 W m^{-2}) depends on the light source (Fig. 5). However, at a high fluence rate (600 W m^{-2}) this dependence was not observed (Fig. 6). At a fluence rate of 40 W m^{-2}, the phage was inactivated to the limit of detection (>99.9999% of inactivation, corresponding to a reduction of 7.2 log) only when irradiated with fluorescent lamps (380–700 nm). With a halogen lamp coupled to an interchangeable fiber optic probe (400–800 nm), the rate of phage inactivation was considerably lower (reduction of 2.5 log) even after 270 min of irradiation (Fig. 5). However, the difference between the two sources was only significant for T0, T30 and T60 (ANOVA, $p < 0.05$ for T0, T30 and T60, and $p > 0.05$ for T90, T180 and T270). At 600 W m^{-2}, the phage was inactivated to the limit of detection (>99.9999% of inactivation, reduction of 7.2 log) after 90 min of irradiation with both light sources (solar irradiation and halogen lamp) (Fig. 6). However, the pattern of inactivation for the two light sources was significantly different for T0 and T30 (ANOVA, $p < 0.05$ for T0 and T30 and $p > 0.05$ for T60, T90 and 120).

Influence of fluence rate using a specific light source on bacteriophage inactivation

The previous results lead us to evaluate how the bacteriophage inactivation would be affected by different fluence rates delivered

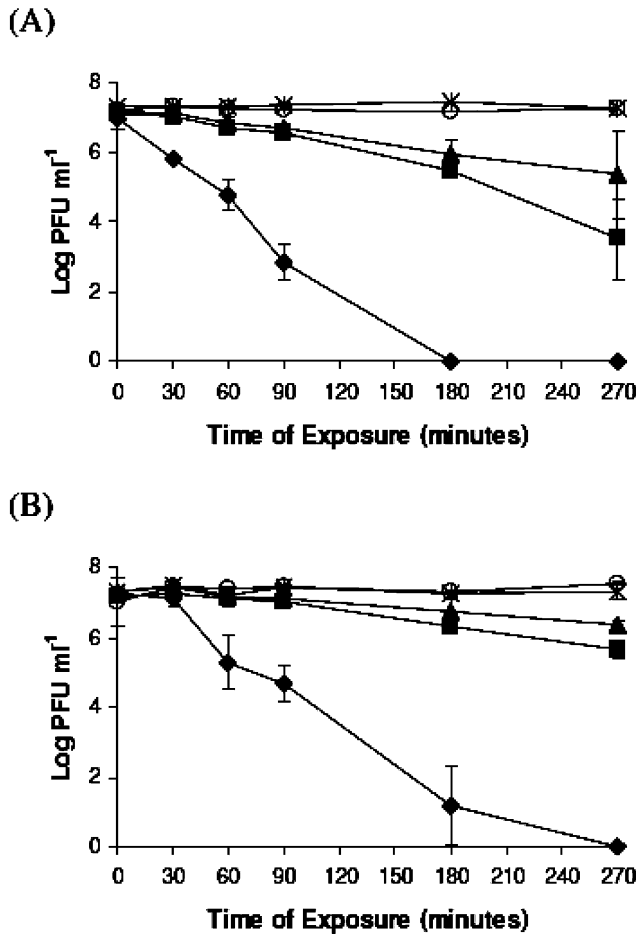

Fig. 4 Density variation of the sewage bacteriophage after irradiation with the a set of 13 fluorescent PAR lamps (40 W m^{-2}) in the presence of Tri-Py^{+}-Me-PF (A) and Tetra-Py^{+}-Me (B), respectively. (○ light control, * dark control, ▲ 0.5 μM, ■ 1.0 μM, ♦ 5.0 μM). Error bars represent standard deviations.

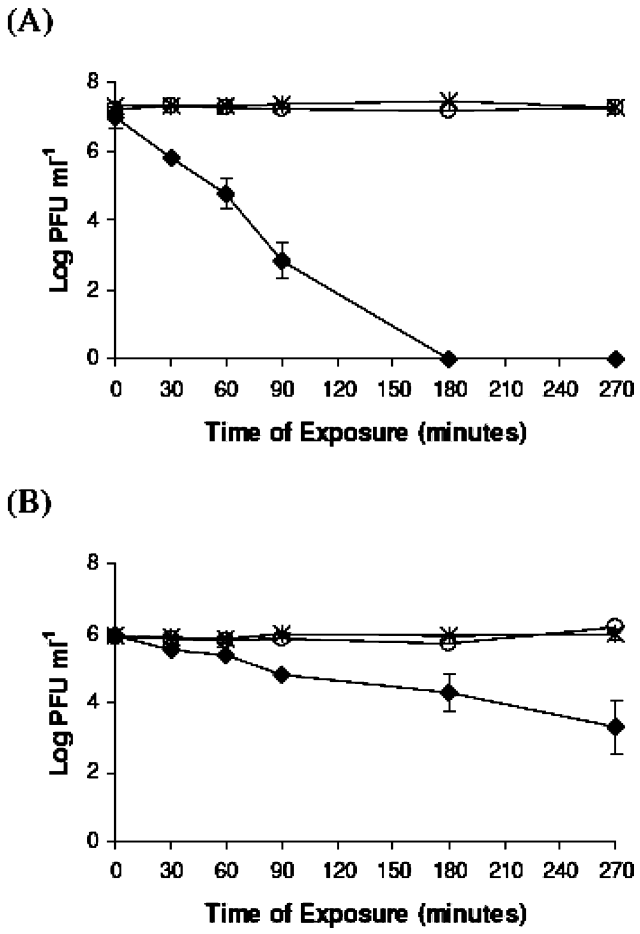

Fig. 5 Density variation of the sewage bacteriophage after irradiation with the a set of 13 fluorescent PAR lamps (A) and illumination system (halogen lamp) (B) at 40 W m^{-2} in the presence of porphyrin Tri-Py^{+}-Me-PF. (○ light control, * dark control, ♦ 5.0 μM). Error bars represent standard deviations.

by the same light source. This is to ensure that the obtained results were due only to the fluence rate, and not to the different emission spectra of the light sources. In this study we used the halogen lamp and the most efficient PS (Tri-Py^{+}-Me-PF).

For a total light dose of 216 J cm^{-2}, the rate of viral photoinactivation was more effective when the fluence rates were 150, 300 and 600 W m^{-2}. Under these conditions, the phage was inactivated to the limit of detection (>99.9999% of inactivation) (Fig. 7). However, for a fluence rate of 1200 W m^{-2} the bacteriophage was not totally inactivated (reduction of 5.4 log). The differences between the phage inactivation with a fluence rate of 1200 W m^{-2} and other fluence rates were significant except for the highest light dose (ANOVA, $p > 0.05$).

For total light doses of 144 and 108 J cm^{-2}, the rate of phage inactivation was higher when the light was delivered at a lower fluence rate (reductions of 5.91 and 4.94 log, with 150 and 300 W m^{-2} for 144 J cm^{-2} and of 3.96 and 3.64 log for 108 J cm^{-2}, respectively) than when the light was delivered at 600 and 1200 W m^{-2} (reductions ranging from 1.77 to 3.09 log). The same pattern was obtained when a light dose of 54 J cm^{-2} was used (reductions of 2.27 and 1.77 log for 150 W m^{-2} and 300 W m^{-2} and of 1.44 and 1.07, respectively for 600 and 1200 W m^{-2}).

Discussion

The present study demonstrates that the two cationic porphyrins Tetra-Py^{+}-Me and Tri-Py^{+}-Me-PF, when irradiated with different sources of light (fluorescent PAR lamps, sun light and halogen lamp) with fluence rates ranging from 40 W m^{-2} to 1690 W m^{-2}, can efficiently photoinactivate sewage non-enveloped viruses. All light sources tested lead to reductions of >99.9999% for the somatic T4-like phage. However, the rate and the extent of inactivation are dependent on: (1) the structure and concentration of the photosensitizer; (2) the light source, namely when low fluence rates are used (40 W m^{-2}); and (3) the energy dose, being considerably more effective when light was delivered at a lower fluence rate.

At the highest concentration tested (5.0 μM) reductions of approximately 7 log are obtained with both porphyrins irrespective of the light source used. However, depending on the light source used, different irradiation periods are required to obtain these reductions. Irradiation with a halogen lamp (1690 W m^{-2}) for 45 min leads to an efficient phage inactivation (~7.2 log of reduction) with both sensitizers. Both PS when irradiated with solar light (600 W m^{-2}) or with a set of fluorescent lamps (40 W m^{-2}) for short periods gave a small phage inactivation (until 2.2 log after 60 min of irradiation) but are able to inactivate the phage to

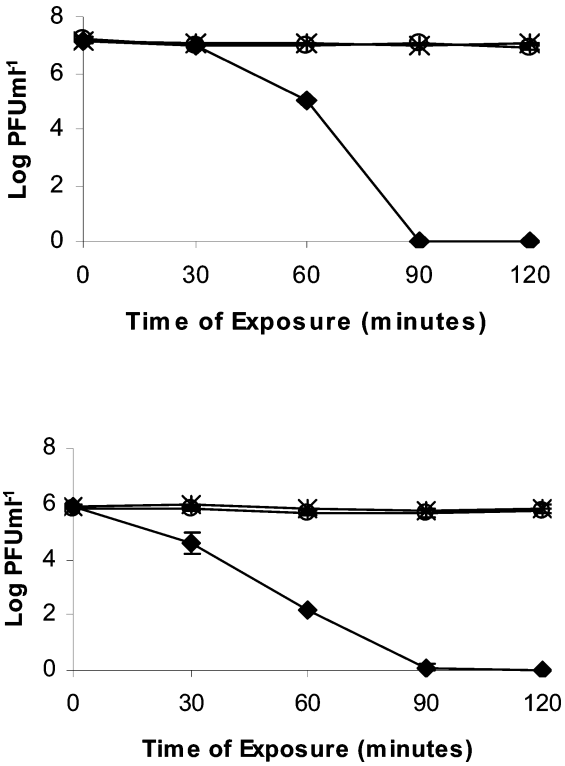

Fig. 6 Density variation of the sewage bacteriophage after irradiation with solar light (A) and with the illumination system (halogen lamp) (B) at 600 W m^{-2} in the presence of porphyrin Tri-Py^{+}-Me-PF. (○ light control, * dark control, ♦ 5.0 μM). Error bars represent standard deviations.

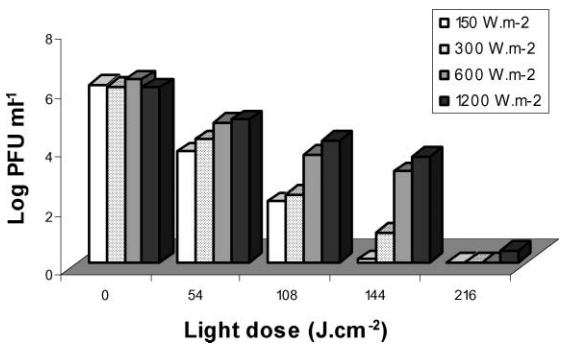

Fig. 7 Density variation of the sewage bacteriophage after a total light dose of 54, 108, 144 and 216 J cm^{-2} delivered at the fluence rates of 150, 300, 600 and 1200 W m^{-2}.

the limit of detection (reductions of about 7 log) after 180–270 min of irradiation. It is important to note, however, that at the end of the experiments, for the lowest photosensitizer concentrations (0.5 and 1.0 μM), the rate of inactivation was higher with solar light and with fluorescent lamps rather than with a halogen lamp. This can be explained by the fact that less PS molecules are available in the microcosms for the photoinactivation process and a portion of the light energy is not used to generate cytotoxic oxygen species.

As observed before, the structure and concentration of the photosensitizer influences phage photoinactivation.[11,20,21,31–33] The two cationic porphyrins, irradiated under the same conditions, inactivated the sewage T4-like phage differently. For concentrations of 5.0 μM, the reductions observed were almost 7 log (limit of detection) for both Tri-Py^{+}-Me-PF and Tetra-Py^{+}-Me although these reductions were reached at different irradiation times. However, at 1.0 and 0.5 μM the rate of inactivation was significantly lower (3.6 and 1.8 log, respectively). In fact, it is known that increasing the concentration of a sensitizer at a fixed light dose leads to increased viral inactivation as does an increase in the total light exposure at a fixed sensitizer concentration.[26] Milanesio and co-workers also support the basic concept that, after irradiation with visible light, cell survival of human cells is dependent upon both intracellular sensitizer concentration and light exposure level.[31] Similar results were obtained by other authors with bacteria.[32,33]

The results also show that the efficacy of sewage T4-like bacteriophage inactivation using the same fluence rate is dependent on the light source used (for Tri-Py^{+}-Me-PF), in particular when the light is delivered at low fluence rate. At 40 W m^{-2} the phage inactivation with fluorescent lamps is much more efficient than with halogen lamps under the same experimental conditions (fluence rate, irradiation time and PS concentration). However, similar phage inactivation is observed when a halogen lamp at 600 W m^{-2} or solar irradiation is used. The difference in phage inactivation when the same fluence rate of 40 W m^{-2} is delivered by the two different light sources can be justified because the fraction of PS that is excited is not the same.[34,35] Although the concentration of the PS used is the same, the emission spectra of the two lamps are different and, consequently, the energy available to excite the photosensitizer is different. The spectral ranges are: 380–700 nm for the fluorescent lamps and 400–800 nm for the interchangeable fiber optic probe coupled to the illumination system (halogen lamp). Since the absorption wavelengths for the porphyrin derivatives range from 400 to 650 nm, part of the energy provided by the halogen lamp (650–800 nm) is not used to excite the photosensitizer. On the contrary, for the fluorescent lamps most of the energy is emitted at 545 and 611 nm that coincides with the Q bands of the PS. When the illumination system (halogen lamp) at 600 W m^{-2} and solar irradiation are used this aspect is not so critical for the inactivation process due to the similarity of both emission spectra (Fig. 8).

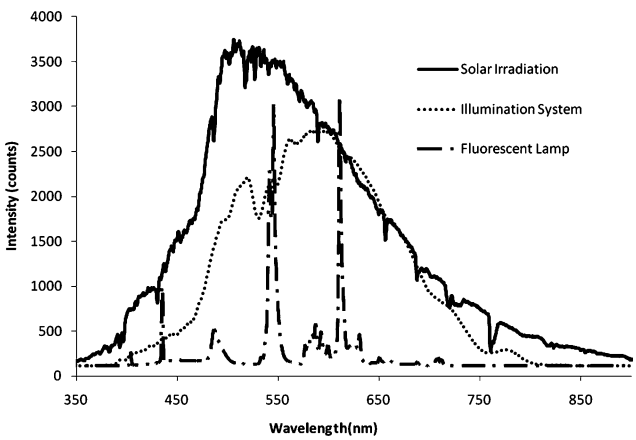

Fig. 8 Emission spectra of light sources used in this study.

When the same light dose is delivered by the same light source but at different fluence rates (150, 300, 600 and 1200 W m^{-2}) the efficacy of sewage T4-like bacteriophage inactivation is inversely proportional to the fluence rate used. Our results show (Fig. 7) that

the photoinactivation is more efficient if the same total amount of photons passing through a bacteriophage suspension is delivered during a longer illumination period (lower fluence rate). This is due to the fact that, when a high fluence rate is used, the PS in the suspension is not able to absorb the photons in excess.[25,36] Similar results were reported by Gábor and collaborators[37] were they described a higher rate of *Escherichia coli* and *Enterococcus hirae* inactivation when a similar total light dose is received over a longer time period. Recently, Prates *et al.* described dissimilar levels of inactivation on yeast cells when the same light dose is given in different fluence rates. At the same light dose, the low fluence rate was more effective than the higher fluence rate.[25]

In conclusion, our results show that cationic porphyrins irradiated with three different light sources are able to efficiently photoinactivate environmental non-enveloped viruses. These compounds can be envisaged as new, cheap and accessible technology for wastewater treatment. All light sources used here were effective in the excitation of the selected PS. Different levels of photoinactivation were achieved when the same light dose is received at different periods of irradiation. At higher doses (216 J cm^{-2}) the efficiency of the photoinactivation process is not significantly affected by the fluence rate, however, at lower light doses, the efficiency is strongly affected by the fluence rate. As the light parameters such as light source, fluence rate and total light dose play an important role in the effectiveness of antimicrobial photodynamic therapy, they should always be considered when establishing an optimal antimicrobial protocol.

Experimental

Porphyrin synthesis

Porphyrins 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (Tetra-Py^+-Me) and 5-(pentafluorophenyl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin tri-iodide (Tri-Py^+-Me-PF) (Fig. 1) used in this work were prepared in two steps as previously described.[38,39] First, the neutral porphyrins were synthesized by Rothemund and crossed Rothemund reactions, using pyrrole and the appropriate benzaldehydes (pyridine-4-carbaldehyde and pentafluorophenylbenzaldehyde) at reflux in acetic acid and nitrobenzene. Then, the resulting porphyrins were purified by column chromatography (silica) and the pyridyl groups were quaternized by reaction with methyl iodide. Porphyrins were purified by crystallization from chloroform-methanol-petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. The spectroscopic data was in accordance with the literature.[38,39] Stock solutions (500 μM) of each porphyrin in dimethyl sulfoxide were prepared by dissolving the adequate amount of the desired porphyrin in a known volume. The absorption spectral features of the PS were the following: [porphyrin] λ_{max} nm (log ε); [Tetra-Py+-Me] in DMSO 425 (5.43), 516 (4.29), 549 (3.77), 588 (3.84), 642 (3.30); [Tri-Py+-Me-PF] in DMSO 422 (5.48), 485 (3.85), 513 (4.30), 545 (3.70), 640 (3.14).

Phage selection and identification

A wastewater sample from a secondary-treated sewage plant of the city of Aveiro (Portugal) was used to select the somatic bacteriophages of *E. coli* C (ATCC 13706).[21] DNA extraction and purification of phage suspension was done using standard techniques.[40] The phage was identified as a T4-like phage that has 82% of homology with the Enterobacteria phage RB43.[21] The nucleotide sequence of the phage has been deposited in the GenBank database under accession n° EU026274.

Bacteriophage host viability test

As bacteria are sensitive to the photosensitizers, the viability of the viral host was evaluated in order to prove that the phage inactivation was only due to the photoinactivation by the photosensitizer and not due to bacterial host inactivation by the porphyrin derivatives. So, accordingly to the procedure described by Costa *et al.*,[21] additional samples were collected in each sampling time after irradiation, with white light delivered by the set fluorescent PAR lamps with a fluence rate of 40 W m^{-2}. Then the samples were washed by ultra-centrifugation at 28 000 *g* for 90 min, at room temperature, to remove the photosensitizer (washed experiments). The photosensitizer-free pellet of phages was re-suspended in PBS buffer, serially diluted and pour plated by the double layer technique, using the agar double layer technique[41] and the aforementioned strain of *E. coli* as host.[21] The results obtained were compared with those resulting from direct spread (non-washed experiments) after irradiation. This bacteriophage host viability test was done at the beginning of the work and only for the most effective porphyrin (Tri-Py^+-Me-PF) at the highest concentration (5.0 μM). In the other experiments this step was not done, but the Petri dishes were incubated in dark conditions.

Light sources

The photodynamic effect of the cationic PS was evaluated by exposing the sewage somatic bacteriophage in the presence of PS to a set of fluorescent PAR lamps, halogen lamp and sun. The first light source is constituted by 13 fluorescent lamps OSRAM 21 of 18 W each one, PAR radiation (380–700 nm) with a fluence rate of 40 W m^{-2}. The second light source used is comprised of an illumination system (LC-122 LumaCare, London) equipped with a halogen 250 W quartz-type lamp and coupled to an interchangeable fiber optic probe (400–800 nm). This illumination system was used to irradiate the microcosm's setup with white light (400–800 nm) at fluence rates from 40 W m^{-2} to 1690 W m^{-2}. The desired fluence rate was obtained by variation of the distance of the fiber tip to the beakers. The experiments with solar irradiation were carried out outside the laboratory. Samples were exposed to solar PAR light on sunny summer days, with an average fluence rate of 600 W m^{-2}. The microcosm was covered with a glass Petri plate to filter the ultraviolet radiation. Only the PAR radiation of the solar spectrum was used in order to avoid viral inactivation by UV radiation during solar exposure and thus to allow the comparison of the results with those obtained with the two artificial lights. UV radiation would inactivate the viruses and, consequently, would increase the rate of phage inactivation during the exposition to the photosensitizers. All the fluence rates were measured with a radiometer LI-COR Model LI-250.

Experiments with different light sources

The influence of different light sources of white light on phage inactivation by the two cationic porphyrins at different

concentrations was evaluated through quantification of the number of bacteriophages in laboratory conditions. A phage suspension was diluted in phosphate buffer (PBS) until 5×10^7 PFU mL^{-1} and aseptically distributed in 600 mL acid-washed and sterilised glass beakers (20 mL per each of five beakers). The photosensitizer from a stock solution (500 μM in DMSO) was added to three beakers to achieve final concentrations of 0.5, 1.0 and 5.0 μM and the other two were used as dark and light controls. In the light control no porphyrin was added but the beaker was exposed to the same irradiation protocol. In the dark control, the photosensitizer at the highest concentration (5.0 μM) was added to the beaker and it was covered with aluminium foil. The test beakers and the light and dark controls were submitted to a pre-irradiation period in the dark during 10 min under 100 rpm stirring at 20–25 °C. After the pre-irradiation period, the 5 beakers, under stirring (100 rpm) and in a thermostatized bath at 20–25 °C, were exposed in parallel to white light radiation generated by the different light sources. Sub-samples of 1.0 mL were taken at regular intervals and analysed, in duplicate, for bacteriophage number. Since the irradiation sources had different fluence rates, it was also compared the efficacy of sewage T4-like bacteriophage inactivation using the same fluence rate delivered by different light sources. These experiments were carried out using the most efficient photosensitizer Tri-Py$^+$-Me-PF at the highest concentration (5.0 μM) irradiated at 40 and 600 W m^{-2}. The Petri plates were kept in the dark immediately after spreading and during the incubation to avoid the inactivation of the bacterial host by the photosensitizer. Viral density (PFU mL^{-1}) was determined at each time point as the mean of the two duplicates in the most convenient dilution series. Viral survival at each time was calculated by dividing the mean number of viruses surviving at each time by the initial number (at time zero). Viral reduction was expressed as a $\log_{10}$ values. For each experimental condition (light source, photosensitizer and concentration) two independent experiments were carried out with two replicates each.

Experiments at different fluence rates with a fixed total light dose

To evaluate the influence of the light fluence rate on the bacteriophage inactivation, phage suspensions, incubated with the most effective porphyrin (Tri-Py$^+$-Me-PF), were exposed to the same energy dose delivered with four different light fluence rates ranging from 150 to 1200 W m^{-2}. An illumination system (LC-122 LumaCare, London) equipped with a halogen 250 W quartz-type lamp and coupled to an interchangeable fiber optic probe (400–800 nm) was used. The fiber tip was placed at the adequate distance from the beaker in order to deliver a fluence rate of 150, 300, 600 and 1200 W m^{-2}. The total light dose of 216 J cm^{-2} corresponded to irradiation periods of 30 min at 1200 W m^{-2}, 60 min at 600 W m^{-2}, 120 min at 300 W m^{-2} and 240 min at 150 W m^{-2}. Sub-samples of 1.0 mL were collected at established intervals corresponding to 54, 108, 144 and 216 J cm^{-2} light doses.

The rate of phage inactivation was evaluated through the quantification of the number of bacteriophages, using the protocol described above. Light and dark control experiments were also carried out simultaneously. For each irradiation period two independent experiments were carried out with two replicates each.

Statistical analysis

All experiments were done in duplicate. Statistical analysis was performed using SPSSWIN 14.0. The significance of difference in phage inactivation between the two photosensitizers' values was assessed using one-way ANOVA. The differences in phage inactivation during the incubation period were also evaluated using one-way ANOVA. Only the data with normal distribution (assessed by Kolmogorov-Smirnov test) and with homogeneity of variances (assessed by Levene test) were used. A value of $p < 0.05$ was considered significant.

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