Applicability of photodynamic antimicrobial chemotherapy as an alternative to inactivate fish pathogenic bacteria in aquaculture systems

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Aquaculture activities are increasing worldwide, stimulated by the progressive reduction of natural fish stocks in the oceans. However, these activities also suffer heavy production and financial losses resulting from fish infections caused by microbial pathogens, including multidrug resistant bacteria. Therefore, strategies to control fish infections are urgently needed, in order to make aquaculture industry more sustainable. Antimicrobial photodynamic therapy (aPDT) has emerged as an alternative to treat diseases and prevent the development of antibiotic resistance by pathogenic bacteria. The aim of this work was to evaluate the applicability of aPDT to inactivate pathogenic fish bacteria. To reach this objective a cationic porphyrin Tri-Py$^+$-Me-PF was tested against nine pathogenic bacteria isolated from a semi-intensive aquaculture system and against the cultivable bacteria of the aquaculture system. The ecological impact of aPDT in the aquatic environment was also tested on the natural bacterial community, using the overall bacterial community structure and the cultivable bacteria as indicators. Photodynamic inactivation of bacterial isolates and of cultivable bacteria was assessed counting the number of colonies. The impact of aPDT in the overall bacterial community structure of the aquaculture water was evaluated by denaturing gel gradient electrophoresis (DGGE). The results showed that, in the presence of Tri-Py$^+$-Me-PF, the growth of bacterial isolates was inhibited, resulting in a decrease of $\approx$7–8 log after 60–270 min of irradiation. Cultivable bacteria were also considerably affected, showing decreases up to the detection limit ($\approx$2 log decrease on cell survival), but the inactivation rate varied significantly with the sampling period. The DGGE fingerprint analyses revealed changes in the bacterial community structure caused by the combination of aPDT and light. The results indicate that aPDT can be regarded as a new approach to control fish infections in aquaculture systems, but it is clearly more difficult to inactivate the complex natural bacterial communities of aquaculture waters than pure cultures of bacteria isolated from aquaculture systems. Considering the use of aPDT to inactivate pathogenic microbial community of aquaculture systems the monitoring of microorganisms is needed in order to select the most effective conditions.

Introduction

Aquaculture provides nearly one-third of the world seafood supplies and is one of the fastest growing agricultural sectors. The growth and even survival of aquaculture industry is, however, threatened by uncontrolled microbial diseases that cause extensive losses.

Bacterial diseases are major problems in the expanding aquaculture industry.[1] There are two broad groups of bacteria of public health significance that contaminate aquaculture products: those naturally present in the environment, the indigenous microflora, and those introduced through environmental contamination by domestic animals excreta and/or human wastes, the non-indigenous microflora.[2] Among these, Gram-negative [Gram (−)] are the major bacterial pathogens that affect various finfish (*Aeromonas salmonicida*, *Aeromonas hydrophila*, *Vibrio anguillarum*, *Edwarsielle tarda*, *Pseudomonas* sp., *Yersinia ruckeri*, *Flavobacterium columnaris*, *Flavobacterium* sp., *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella*), *Photobacterium damselae* subsp. *damselae* (formerly *Vibrio damsela*).[3–6] On the other hand, only a few Gram-positive [Gram (+)] species affect finfish, such as *Renibacterium salmoninarum*, *Nocardia* spp., *Mycobacterium* sp., *Streptococcus* sp.[3–5] The increasing problems with worldwide emergence of multidrug-resistant strains among common pathogenic bacteria and the concern about antibiotics

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spreading in the environment due to medical treatments in humans, in animal farms and in fish farms, bring about the need to find new methods to control fish pathogens.

Although disease prevention is the preferred approach and the most cost-effective in the aquaculture industry, control of infections is difficult in fish-farming conditions.[7] Poor water quality, ubiquitous nature and rapid spreading of pathogens, environmental adverse conditions, high stocking densities, different stages of the fish life cycle, resistance in common pathogenic bacteria, low activity of chemotherapeutic agents against bacterial endospores and fungal zoospores, and few drugs licensed for fishery use are factors that make disease prevention difficult in aquaculture.[6]

Presently, antimicrobial photodynamic therapy (aPDT) is receiving considerable attention for its potential as a new form of antimicrobial treatment.[8–11] It has been proved that microorganisms do not develop resistance to aPDT.[12–14] Some authors used aPDT to inactivate drug-resistant bacteria and have found that these bacteria are as susceptible as their native counterparts to aPDT technology.[12–16] Consequently, aPDT seems to be a potential alternative for the treatment and eradication of microbial infections. The aPDT approach is based on the photodynamic therapy concept, in which a photosensitizer (PS) localized preferentially in target cells, when activated by low doses of visible light at an appropriate wavelength, generates cytotoxic species (reactive oxygen species such as singlet oxygen and free radicals) that will destroy or damage biological molecules by oxidation.[8,17,18]

Currently, the major use of aPDT is in the clinical area[8,10,11,19–21] but recent studies have shown that photocatalytic therapy for microbial inactivation in drinking[22] and wastewaters[23–26] is also possible and that solar radiation can be used as a light source. Although there is only one study of aPDT in aquaculture systems, preliminary results indicate that this technology has a high potential to disinfect aquaculture waters. The results of this study show that aPDT approach is effective to treat saprolegniosis of infected fish farming stocks (6–7 log decrease on cell survival) without perilesional damage of the fish or recurrence of the disease in infected sites or in other fish sites.[27,28] Wong *et al.* (2005) showed also that *Vibrio vulnificus*, which frequently infects fish and contaminates fish farming waters, is inactivated by aPDT.[28] Moreover, the possibility of using PS immobilized in insoluble solid supports allows the retention of the PS after the photoinactivation (PI) process, avoiding its release to the environment and allowing its reuse, making the aPDT approach cost-effective and environmentally friendly.[6,22,29–31]

The effective inactivation of microorganisms, the improbable development of photo-resistant strains, and the possibility of irradiating fish-farming waters in the presence of immobilized PS using solar light, suggest that aPDT can be considered an alternative technology to disinfect aquaculture waters. However, as the photosensitisers do not present microbial selectivity, they can, consequently, inactivate non-pathogenic microorganisms that can have an important ecological role in aquatic systems, like in intensive and semi-intensive aquaculture systems. These bacteria have a central role in the functioning and productivity of the aquatic systems. They are the most important biological component involved in the turnover (transformation and remineralization) of organic matter in aquatic systems.[32,33] In coastal waters, heterotrophic bacteria may utilize as much as

40% of the carbon fixed by the primary producers[32,34,35] and are particularly skilled for organic matter transformation. As bacteria are the major players in the biogeochemical cycles in the aquatic environment[32,36,37] they represent a good choice to evaluate the impact of aPDT. As only a small fraction of bacteria in the aquatic system is active[38] the measurement of the total number of bacteria (active and inactive bacteria) is not a good option to evaluate the impact of this approach in the ecosystem. The fraction of cultivable bacteria, which includes most of the known fish pathogenic bacteria, corresponds only to active bacteria, being an appropriate indicator to evaluate the impact of the aPDT process in the aquaculture systems. Nonetheless, as a large fraction of bacteria is non-cultivable, and thus not detected by traditional methods, molecular tools (as denaturating gradient gel electrophoresis, DGGE) must be used to evaluate the effect of aPDT on the overall bacterial community.[39]

The aim of this work was to evaluate the applicability of aPDT to inactivate fish pathogenic bacteria. To reach this objective a cationic porphyrin derivative 5,10,15-tris(1-methylpyridinium-4-yl)-20-pentafluorophenylporphyrin tri-iodide (Tri-Py$^+$-Me-PF), was tested against nine pathogenic bacteria (*Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Aeromonas salmonicida*, *Photobacterium damselae* subsp. *damselae*, *Photobacterium damselae* subsp. *piscicida*, *Escherichia coli*, *Pseudomonas* sp., *Enterococcus faecalis* and *Staphylococcus aureus*). In order to infer the ecological impact of aPDT in the aquatic environment, the aPDT effect was also tested directly on the natural bacterial community. The effect of aPDT on the density of the cultivable bacteria fraction and on the overall bacterial community structural diversity was evaluated.

Results

Inactivation of isolated bacteria from aquaculture water

The results obtained from the aPDT against the nine isolated bacteria under study are summarized in Fig. 1 for the Gram (−) strains (*V. anguillarum*, *V. parahaemolyticus*, *A. salmonicida*, *P. damselae* subsp. *damselae*, *P. damselae* subsp. *piscicida*, *E. coli*, *Pseudomonas* sp.) and in Fig. 2 for the Gram (+) strains (*S. aureus* and *E. faecalis*).

According to the experimental data, the porphyrin Tri-Py$^+$-Me-PF was effective against all the bacteria Gram (−) and Gram (+) strains, causing, in most cases, approximately 8 log reduction on cell survival, after 270 min of exposure to white light, with a fluence rate of 40 W m^{-2}. The PI rate of Gram (−) bacteria in comparison to Gram (+) bacteria was slower (Fig. 1 and Fig. 2).

With the exception of *A. salmonicida*, all the Gram (−) strains were inactivated to the limits of detection, but the pattern of inactivation during the irradiation period varied with the bacterial strain (Fig. 1) (ANOVA $p < 0.05$).

For the two strains of *Photobacterium*, the major reduction on cell viability occurred after 15 min of irradiation, causing a decrease of approximately 5.7 log (Fig. 1A and 1B). The PI process against *P. damselae* subsp. *damselae* shows a decrease on the cell viability of ~7.9 log after 90 min of irradiation, but for *P. damselae* subsp. *piscicida* a similar effect on cell viability (~8.0 log decrease) occurs only after 180 min of irradiation (Fig. 1A and 1B). For the two strains of *Vibrio* (Fig. 1C and 1D), the major reduction on cell viability occurred after 30 min of irradiation for
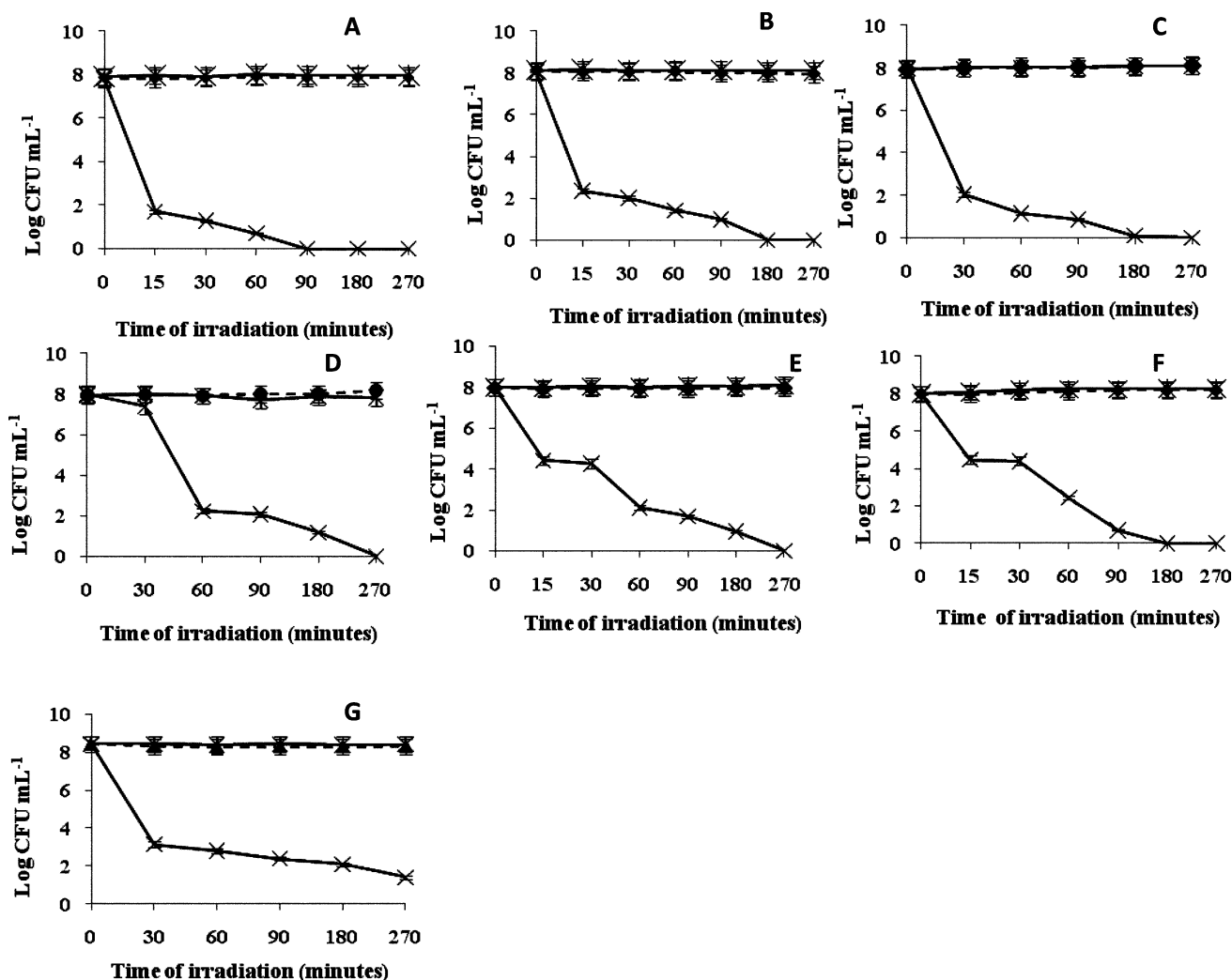
Fig. 1 Variation on viability of *P. damselae* subsp. *damselae* (A), *P. damselae* subsp. *piscicida* (B), *V. parahaemolyticus* (C), *V. anguillarum* (D), *Pseudomonas* sp. (E), *E. coli* (F) and *A. salmonicida* (G) isolated from aquaculture water in the presence of Tri-Py$^+$-Me-PF and exposed to artificial white light (40 W m^{-2}) during 270 min. Each value represents mean ± standard deviation (sometimes is hidden under the symbols) of two independent experiments. (—✕—) 5.0 μM of Tri-Py$^+$-Me-PF, (--◆--) dark control with 5.0 μM of Tri-Py$^+$-Me-PF, (—✱—) light control without Tri-Py$^+$-Me-PF.
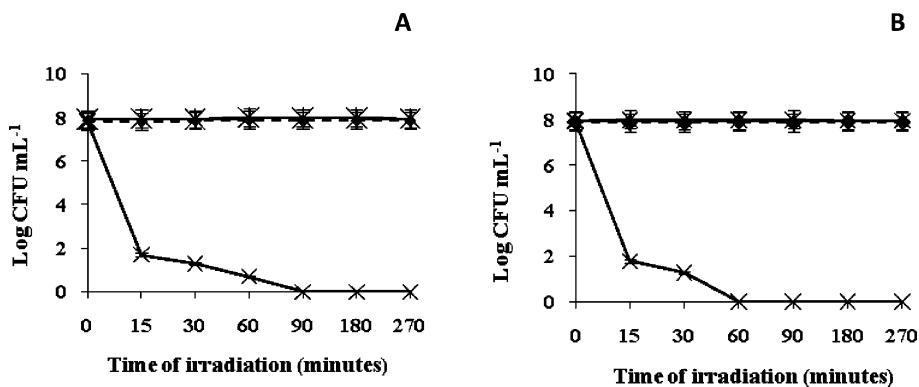
Fig. 2 Variation on viability of *E. faecalis* (A) and *S. aureus* (B), isolated from aquaculture water in the presence of Tri-Py$^+$-Me-PF and exposed to artificial white light (40 W m^{-2}) during 15, 30, 60, 90, 180 and 270 min. Each value represents mean ± standard deviation (sometimes is hidden under the symbols) of two independent experiments. (—✕—) 5.0 μM of Tri-Py$^+$-Me-PF, (--◆--) 5.0 μM of Tri-Py$^+$-Me-PF dark control, (—✱—) light control without Tri-Py$^+$-Me-PF.

V. parahaemolyticus (5.9 log of reduction) and after 60 min of irradiation for *V. anguillarum* (~5.8 log reduction). After 180 and 270 min of exposure to white light reductions of ~7.5 log and of 8.0 log on cell viability of *V. anguillarum* and of *V. parahaemolyticus* were obtained, respectively. The Tri-Py$^+$-Me-PF was also a good PS against *Pseudomonas* sp. and *E. coli*. In fact, a reduction of 8.0 log on *Pseudomonas* sp. viability after 270 min of irradiation was observed (Fig. 1E) as well as a similar reduction on *E. coli* (8.0 log) after 180 min of irradiation (Fig. 1F). The major reduction on cell viability for these two bacteria, causing approximately 4 log decrease, was obtained after 15 min of irradiation. The viability of *A. salmonicida* was also affected by the PI process but this bacteria was not inactivated to the detection limit after the 270 min of irradiation. However, reductions of 5.3 log and 7.0 log were obtained after 30 and 270 min of irradiation, respectively (Fig. 1G).

The Gram (+) strains were more easily photoinactivated than Gram (−), showing a rapid decrease on cell survival with the PS used (Fig. 2). A reduction on *E. faecalis* viability to the detection limit after 90 min of irradiation (7.8 log reduction) (Fig. 2A). Results for *S. aureus* showed a reduction of 8.0 log after 60 min of irradiation (Fig. 2B). The major reduction on cell viability for both strains was obtained after 15 min of irradiation causing approximately 6 log of reduction.

The results of light and dark controls showed that the sole exposure to light or to the PS did not significantly affect the cell viability (Fig. 1 and 2). This indicates that the reduction on cell survival was due to the PI process.

Inactivation of aquaculture water cultivable bacteria

The efficiency of the Tri-Py$^+$-Me-PF to inactivate the cultivable bacteria community was studied using water samples collected at four different dates in 2009 (26th March, 6th May, 21th May and 26th June) and in June 8th, 2010. The results show that the effectiveness of the Tri-Py$^+$-Me-PF to inactivate the cultivable bacteria community varied during the sampling period (Fig. 3).
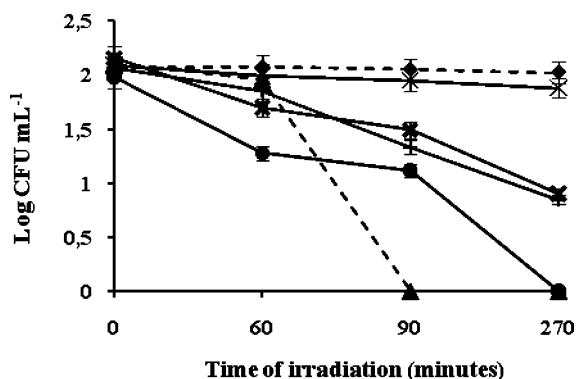

Fig. 3 Logarithmic reduction of cultivable bacterial density, in the presence of Tri-Py$^+$-Me-PF and exposed to artificial white light (40 W m^{-2}) during 270 min collecting on 4 different days in 2009: March 26th (−▲−), May 6th (−✕−), May 21th (−+−) and June 26th (−●−), light control without Tri-Py$^+$-Me-PF (−✳−), dark control 5.0 μM of Tri-Py$^+$-Me-PF (--◆--).

In samples collected on March 26th and June 26th, cells were inactivated to the detection limit (2 log reduction) after 90 and 270 min of irradiation, respectively (Fig. 3) with 5.0 μM.

The bacterial inactivation in samples collected on May 6th and 21st were less effective, showing a 1.3 log and 1.2 log reduction on cell viability, respectively, after 270 min of irradiation at 5.0 μM (Fig. 3). Light and dark controls did not show a significant reduction during the irradiation time, indicating that the reduction on cell viability after irradiation of treated samples was due to the photodynamic effect of the PS.

Effect of aPDT on the bacterial community structure

A water sample was collected on 8th June 2010 in order to study the effect of the photodynamic process, due to the Tri-Py$^+$-Me-PF, on the total bacterial community structure of the aquaculture system by DGGE after PCR-amplification of 16S rRNA (cDNA) fragments. In contrast to DNA, short-lived RNA molecules are degraded rapidly in living bacterial cells by enzymes (RNase), which are very stable even in harsh environments.[40,41] Therefore, specific RNA sequences represent an excellent target molecule for the detection of rapid changes in bacterial communities. The DGGE profile resulting from separation of cDNA fragments of 16S rRNA amplified by PCR revealed a complex pattern of bands (Fig. 4) in the bacterial community of the aquaculture plant. In the presence of Tri-Py$^+$-Me-PF, irradiation for 270 min (PT$_{270}$) leads to a reduction in the number of bands detected in DGGE profiles when compared with the initial community at time 0 (LCT$_0$). In fact, the statistical analysis confirmed that the bacterial community of the aquaculture after 270 min of irradiation in the presence of porphyrin Tri-Py$^+$-Me-PF (PT$_{270}$) and the light control (LCT$_0$) differed significantly (ANOSIM $R = 0.704$).
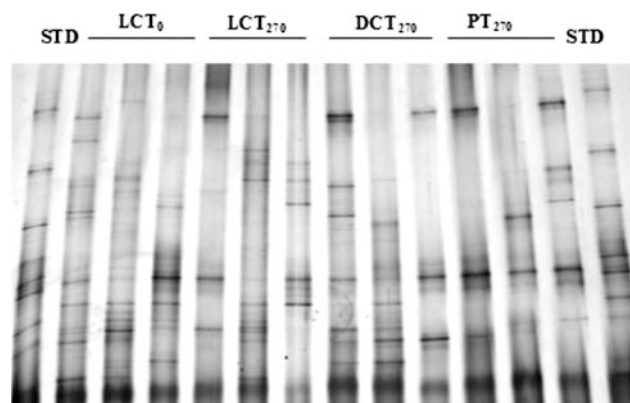

Fig. 4 DGGE profiles of 16S rRNA (cDNA) fragments of bacterial community of the aquaculture system collected at 2010 June 8th in the presence of PS and irradiated with artificial white light (40 W m^{-2}). STD – molecular marker, LCT$_0$ – light control without Tri-Py$^+$-Me-PF at time zero; LCT$_{270}$ – light control without Tri-Py$^+$-Me-PF irradiated for 270 min; DCT$_{270}$ – 5.0 μM of Tri-Py$^+$-Me-PF dark control incubated during 270 min, PT$_{270}$ – sample with 5.0 μM Tri-Py+-Me-PF irradiated for 270 min.

Water samples just exposed to white-light for 270 min (LCT$_{270}$) resulted in a small alteration of the total bacterial community when compared with dark control incubated during the same period (DCT$_{270}$) (ANOSIM $R = 0.370$). Surprisingly, the comparison of the water samples exposure to white-light (LCT$_{270}$) and to

Tri-Py⁺-Me-PF at 5.0 μM and irradiated during 270 min (PT$_{270}$), resulted in low separation between the bacterial community profiles (ANOSIM $R = 0.259$).

These results prompt us to re-evaluate the PI effect of the PS on the cultivable bacteria present in this water sample (June 8th 2010). The amount of cultivable bacteria fraction of this sample was much higher (~3.5 log) than the one observed in the samples collected in 2009 (Fig. 5). The results show that in this case the effectiveness of the Tri-Py⁺-Me-PF at 5.0 μM after exposure to white light during 270 min was very low (less than 1 log reduction). The increase of Tri-Py⁺-Me-PF concentration to 10, 15 and 20 μM, revealed a moderate increase of PI efficiency. Reductions of 1.5 log or less were achieved at PS concentration of 10 and 20 μM after 270 min of irradiation. In fact, the inactivation was only effective at 50 μM where the inactivation reached the detection limit (3.5 log reduction) after 270 min of irradiation (Fig. 5).
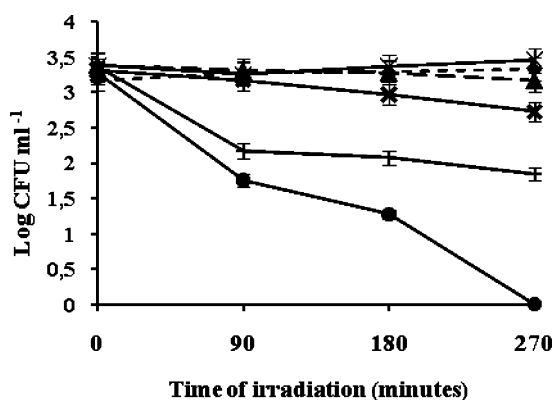
Fig. 5 Logarithmic reduction of cultivable bacterial density, in the presence of Tri-Py⁺-Me-PF and exposed to artificial white light (40 W m⁻²) for 270 min at different concentrations: 5.0 μM (–▲–·), 10 μM (—✕—), 20 μM (—+—) and 50 μM (—●—), light control without Tri-Py⁺-Me-PF (—✳—), dark control with 5 μM of Tri-Py⁺-Me-PF (--◆--).

Discussion

The results obtained in this study indicate that the combination of porphyrin Tri-Py⁺-Me-PF and visible light can represent a viable and environmentally friendly alternative for the control of potentially pathogenic bacteria in aquaculture systems. Moreover, the effectiveness of aPDT in environmental waters varies during the sampling period and to reach an effective inactivation it is necessary to adjust the PS concentration to the cultivable bacteria density.

In this work, nine potential pathogenic bacteria isolated from an aquaculture systems were efficiently photoinactivated using a low concentration of porphyrin (5.0 μM). In general, pathogenic bacteria were inactivated to the detection limits (≈8 log) but the profile of the photoinactivation process varied among the bacterial strain (ANOVA, $p < 0.05$). As previously reported[9,18,42–44] the Gram (+) bacteria were inactivated faster than Gram (−) bacteria. The major reduction on cell viability occurred after 15 min of irradiation, causing approximately 5.5 log decrease for both Gram (+) bacteria, but, in general, the major reduction for Gram (−) bacteria occurred only after 30–60 min of irradiation, causing approximately a 4–6 log decrease. All the Gram (+) and Gram

(−) strains, with the exception of *A. salmonicida*, were completely inactivated after 270 min of exposure to the white light. For *A. salmonicida*, after 270 min of exposure, around 1 log of cell survived to the photoinactivation process.

The photodynamic effect produces damage mainly in the external bacterial structures and in DNA.[18] The damage to the external structures can involve leakage of cellular contents or inactivation of membrane transport systems and enzymes.[45,46] Some damage produced in the DNA can be repaired by the action of DNA-repairing systems.[47] It has been concluded that, although nucleic acid damage occurs, it cannot be the principal cause of microbial photodynamic inactivation.[18,48,49] The difference in the susceptibility of Gram (+) and Gram (−) bacteria to the photoinactivation process is easily explained by the cell wall composition differences.[9,18,42–44] The different profiles of inactivation among the Gram (−) bacteria can be also associated with differences in the cell wall. Although most of the Gram (−) bacteria have a thin layer of peptidoglycan and an outer membrane, the composition of the outer membrane varies among bacteria.[9,42,48,50] Other studies also showed that effectiveness of aPDT depends on the bacterial strain.[51,52]

As is well known, the cell wall of virulent strains of *A. salmonicida* possesses a surface S-layer composed by protein A units arranged tetragonally and contiguously over the cell surface.[53,54] Kay *et al.* (1985)[55] show that *A. salmonicida* protein A has a hydrophobic binding domain with less affinity to hematoporphyrin, containing hydroxyl groups, than for protoporphyrin IX with vinyl groups. The presence of this S-layer in *A. salmonicida* bacteria can probably explain the lower aPDT efficiency relative to the other Gram (−) bacteria when the hydrophilic cationic porphyrin Tri-Py⁺-Me-PF is used. Nevertheless this isolate is not more resistant to antibiotics than the other Gram (−) isolates tested in this study.[56] Further studies are necessary to clarify the underlying mechanisms of the photoinactivation process of the Gram (−) bacteria *A. salmonicida*.

The results of this study indicate that the cultivable fraction of the heterotrophic bacteria of the aquaculture plant was inactivated by the photodynamic process, but its efficiency varied during the sampling period. The cultivable bacteria in a concentration of around 100 CFU mL⁻¹ were more efficiently inactivated in samples collected in March and June 2009 (2 log reduction on cell survival) relative to the two samples collected in May 2009 (1.2 log reduction on cell survival) using 5.0 μM of PS. In June 2010, the higher concentration of viable bacteria (around 1500 CFU) is probably the main responsible for PS higher concentration needed (50 μM) to fully inactivate the bacteria community. In fact, previous studies confirmed the dependence of PI effectiveness with the cell density in the suspension.[42]

In addition, the structure of bacterial community is affected by environmental conditions[57] and a clear pattern of seasonal variation on the structure of bacterial communities has been observed[56,58] in aquaculture systems even for pathogenic bacteria.[56] Consequently, the seasonal variation of photoinactivation observed for the cultivable bacteria can be explained, in part, due to differences in bacterial community structure.

DGGE profiles showed that not all bacterial populations are affected by aPDT (Fig. 4). The band position and relative intensity were not altered for some specific populations. For water samples treated with PS and exposed to light during 270 min, a reduction

on the number of bands relatively to the non-treated water samples was observed. This seems to indicate that dominant bacterial populations were affected by the PS. However, the results obtained from the simple exposure to light also affects the bacterial population. So, under these conditions a high similarity on the bacterial community profile between the experiment performed just under light (LC$_{270}$) and in the presence of PS (PT$_{270}$) is found (ANOSIM $R = 0.259$). These results are corroborated by the poor logaritm reduction of cultivable bacteria density found in this water sample at PS concentration of 5.0 µM. Nevertheless, a better evaluation of the impact of aPDT on bacterial communities of aquaculture systems requires the identification of the most affected bacterial groups, for instance by sequencing.

The results of this study show clearly that it is more difficult to inactivate the complex natural bacterial communities of aquaculture waters (~1×10^6 bacterial cells per mL)[56] than pure cultures of bacterial isolates obtained from aquaculture systems (~1×10^8 cells per mL). Non-target microorganisms such as viruses, fungi and protozoa, and even algae cells present in the water, can also compete for the ROS produced. In fact, the number of viruses in the aquaculture water used in this study reached high concentrations (~1×10^9 particles per mL).[56]

The dissolved and particulate organic matter can also compete with the cultivable bacteria, and with other microorganisms, for the PS, decreasing the real concentration of PS available for their photoinactivation.

As aPDT is not selective for pathogenic microorganisms, the non-pathogenic microbial community of semi-intensive aquaculture systems can also be affected. As non-pathogenic bacteria have an important ecological role in the biogeochemical cycles in these aquaculture systems, a careful evaluation of the environmental impacts must be conducted before aPDT is implemented in these semi-intensive aquacultures. It would be crucial to realize how the non-pathogenic groups of bacteria with a relevant role in the turnover of organic matter in the semi-intensive aquaculture systems are affected by the photodynamic process. However, as the water is renovated at each tidal cycle in semi-intensive aquaculture systems, the non-pathogenic groups of bacteria with a relevant role in the turnover of organic matter would be returned with the incoming water from the estuarine system.

Besides the concern with the disturbance of the balance between microbial communities, the affordability of this technology to the commercial aquaculture producers must be also considered. The idea of this approach is to use solar light and functional cationic nanomagnet-porphyrin hybrids to disinfect the water from aquacultures. The immobilization of the porphyrin allows its recovery and reuse, avoiding ingestion by fish and also the release to the water output, making this technology cost effective and environmentally friendly.

Conclusions

In conclusion, aPDT is effective to inactivate potential bacterial fish pathogens, being a promising alternative approach to traditional methods of disinfection. With aPDT it is able to inactivate 9 bacteria strains isolated from a semi-intensive aquaculture system of Ria de Aveiro (Portugal). The results show that it is possible to photoinactivate natural bacterial communities, but the photoinactivation efficiency is dependent on the seasonal

variation of microbial communities and maybe also on other non-microbial particles. The aPDT technology can be used for water disinfection of the fish tanks, before or after (in case of water recirculation) fish contact. Considering the use of aPDT to inactivate pathogenic microbial community of semi-intensive aquaculture systems, the monitoring of microorganisms (e.g. bacterial community dynamics, abundance and specific bacteria strains) is needed in order to select the most effective conditions. The Tri-Py$^+$-Me-PF has adequate features to be immobilized in solid supports through the pentafluorophenyl group, avoiding its release in the environment and allowing its recovery and reutilization.

Experimental

Photosensitizers

5,10,15-Tris(1-methylpyridinium-4-yl)-20-pentafluorophenyl-porphyrin tri-iodide (Tri-Py$^+$-Me-PF) (Fig. 6) used in this work was prepared according to the literature.[59,60] Porphyrin was purified by crystallization from chloroform/methanol/petroleum ether and their purities were confirmed by thin layer chromatography and by ^{1}H NMR spectroscopy. A stock solution of Tri-Py$^+$-Me-PF in DMSO at 500 µM was prepared, divided into aliquots of 1.5 mL and maintained at 4 °C. Structural characterization and absorption spectral features of the Tri-Py$^+$-Me-PF: ^{1}H NMR: (CDCl$_3$) δ: −3.13 (2H, s, NH), 4.72 (9H, s, CH$_3$), 8.99–9.04, 9.15–9.21, 9.47–9.19 (20H, 3 m, o-Py-H, m-Py-H e β-H). ^{19}F RMN (CDCl$_3$) δ: −158.38 to −158.44 (2F, m, Ar-o-F), −172.51 (1F, t, Ar-p-F), 181.25 to −181.39 (2F, m, Ar-m-F). MS-MALDI TOF/TOF m/z 753 [M+H]$^+$; UV-Vis in DMSO λ_{max} (log ε) 422 (5.48), 485 (3.85), 513 (4.30), 545 (3.70), 640 (3.14) nm. Before each PI assay,
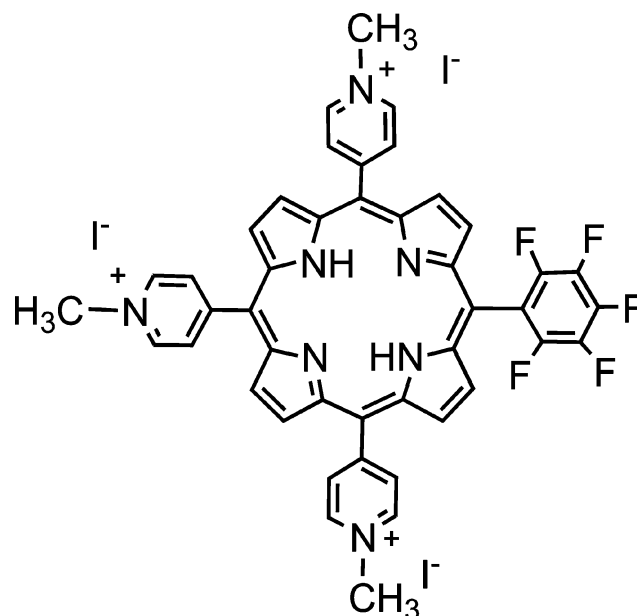
Tri-Py$^+$-Me-PF

Fig. 6 Structure of the PS used in this study.

the porphyrin aliquot to be used was stirred at 120 rpm, until room temperature (25 °C) was reached.

Microorganisms and growth conditions

The Tri-Py$^+$-Me-PF was tested on isolated Gram (+) and Gram (−) bacteria and on the bacterial community of an aquaculture system. Seven of the nine bacterial strains used (*V. anguillarum*, *V. parahaemolyticus*, *A. salmonicida*, *Pseudomonas* sp., *E. coli*, *E. faecalis* and *S. aureus*) were previously isolated in our laboratory from the semi-intensive aquaculture system (Corte das Freiras) of Ria de Aveiro (Portugal).[56] The other two strains *P. damselae damselae* (ATCC 33539) and *P. damselae piscicida* (ATCC 29690) were obtained in previous studies.[61] The bacteria were stored at 4 °C in tryptic soy agar (TSA, Merck). Before each assay the strains were grown aerobically for 24 h at 25 °C (*V. anguillarum*, *V. parahaemolyticus*, *A. salmonicida*, *P. damselae* subsp. *damselae*, *P. damselae* subsp. *piscicida* and *Pseudomonas* sp.) or 37 °C (*E. coli*, *E. faecalis* and *S. aureus*) in 30 mL of tryptic soy broth (TSB, Merck). Then aliquots of these cultures (300 µL) were aseptically subcultured to 30 mL of fresh TSB medium and grew overnight at 25 °C or 37 °C, depending on the bacterium.

Irradiation conditions

The effect of cationic porphyrin Tri-Py$^+$-Me-PF was evaluated by exposing the samples in parallel to white light (PAR radiation, 380–700 nm, 13 OSRAM 21 lamps of 18 W each) with a fluence rate of 40 W m^{-2} (measured with a radiometer Li-COR Model LI-250), at 25 °C or 37 °C for 270 min, under 100 rpm mechanical stirring.

Experimental Setup

Experiments with suspensions of isolated bacteria. The efficiency of the cationic porphyrin Tri-Py$^+$-Me-PF at a concentration of 5.0 µM was evaluated through quantification of the number of colony forming units (CFU) in laboratory conditions. All the experiments were performed using the same experimental conditions and phosphate buffered saline (PBS) was the dilution medium chosen. Overnight bacterial cultures were diluted tenfold in PBS, pH 7.4, to a final concentration of ≈10^8 CFU mL^{-1}. In all experiments, 63 mL of bacterial suspension were aseptically distributed in three 600 mL acid-washed and sterilized glass goblets (20 mL per each goblet). To one goblet (test goblet) the PS was added from the stock solution (500 µM in DMSO) to achieve the final concentration of 5.0 µM and the other two goblets were used as dark and light controls. In the light control, the bacterial suspension without PS was exposed to light irradiation. In the dark control, the PS was added (to achieve a final concentration of 5.0 µM) to the goblet, containing the cell suspension, covered with aluminium foil to protect from light exposure. After the addition of the appropriate volume of porphyrin, goblets were incubated during 10 min at 20–25 °C, under stirring (100 rpm), covered with aluminium foil to avoid accidental light exposure. After this period, all goblets were exposed to white light, at 25 or 37 °C, during 270 min under stirring (100 rpm). A volume of 1.0 mL of test and control samples was collected at time 0 and after pre-defined times (30, 60, 90, 180 and 270 min) of light exposure, then serially diluted and plated in duplicate in TSA medium. The Petri plates were kept on the dark immediately after plating and during the incubation period. After 24 h of incubation at 25 °C or 37 °C, the number of colonies was counted in the most convenient series of dilution. For each bacteria strain two independent experiments were done and the results presented are the average of the two assays.

Statistical analysis was performed using SPSSWIN 14.0. The significance of difference among bacterial inactivation was assessed using one-way ANOVA. The differences in bacteria 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.

Effect of aPDT on the cultivable bacterial density of aquaculture water. Water samples were collected at early morning, two hours before low tide, in mild weather conditions, from the culture tank of *Sparus aurata* at 0.2 m from the surface in different dates: 26th March 2009, 6th May 2009, 21st May 2009, 26th June 2009 and 8th June 2010. The sampling at fixed time during the tidal cycle allows comparison of the results during the sampling period, because during a tidal cycle the microbiological parameters in Ria de Aveiro vary significantly.[62]

Water samples were protected from light and analyzed 1–2 h after collection. The samples were aseptically distributed in three 600 mL acid-washed and sterilized glass goblets (15 mL per each goblet). The PS at 5.0 µM was added to one goblet (test goblet) and the other two goblets were used as light and dark controls. The light control goblet was not added with PS and was exposed to light and the dark control was added of 5.0 µM of PS and protected from light. After the addition of the appropriate volume of PS, all goblets were subjected to the same incubation period prior to irradiation, as mentioned above, at the same conditions. Then, the procedure was the same as for the experiments with suspensions of isolated bacteria. Water samples of 1 mL were collected from each goblet at time 0 and after 60, 90 and 270 min of light exposure; serial dilutions and plating in duplicate in TSA medium. After 3 days of incubation at 25 °C in the dark, the number of colonies was counted on the most convenient series of dilution. On 8th June 2010 a range of PS concentrations was tested (5, 10, 20 and 50 µM).

Effect of aPDT on the bacterial community structure. Water samples were collected from the aquaculture plant two hours before low tide at 0.2 m from the surface in the culture tank of *Sparus aurata* on 8th June 2010. Water samples were protected from light and analyzed 1–2 h after collection. For the characterization of the bacterial community structure three independent experiments were done for each experimental condition. One hundred and fifty millilitres of water were aseptically distributed in nine 600 mL acid-washed and sterilized glass goblets (3 test goblets, 3 light controls, LC, and 3 dark controls, DC). In the test goblets, an appropriate volume of Tri-Py$^+$-Me-PF was added to reach the concentration of 5.0 µM (final concentration) (P). Light (LC) and dark (DC) were prepared as mentioned above. The test, light and dark control goblets were exposed to white light (40 W m^{-2}), at 25 °C, during 270 min under stirring (100 rpm). Three other water samples of 150 mL were used to characterize the natural bacterial communities of the water, these samples were not

exposed to white light. Afterwards, all the 12 samples were filtered through 0.2 μm pore-size filters (Poretics Products, Livermore, USA). For RNA extraction, the bacterial cells retained on the membranes were resuspended in 2 mL of TE buffer [10mM Tris HCl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] and centrifuged. RNA was isolated using the UltraClean Microbial RNA kit (Mo Bio Laboratories, Carlsbad, CA), according to the instructions provided by the manufacturer. The RNA solution was treated with 20 U RNase-free DNase to remove any traces of DNA. The efficiency of the DNA removal from RNA was checked as described by Moeseneder et al. (2001).[62] First strand cDNA synthesis was conducted using Maxima™ Reverse Transcriptase (Fermentas) in 20 μL reaction mixtures containing 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂, 50 mM dithiothreitol, deoxynucleoside triphosphates each at a concentration of 0.5 mM, 100 pmol of random primer, 20 U of RiboLock™ RNase inhibitor and 20 U of reverse transcriptase. The reactions were performed for 10 min at 25 °C, followed by incubation at 50 °C for 30 min, and a final 5 min incubation at 85 °C to inactivate the reverse transcriptase. Reverse transcriptase products were kept frozen at −20 °C until use. The RT products were used to amplify 16 rRNA (cDNA) fragments, using a nested PCR approach. The reaction was carried in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) from MIDSCI. In the first PCR, the universal bacterial primers 27F and 1494R were used to amplify ca. 1450 bp of the 16S rRNA[63] A reaction mixture of 25 μL was prepared containing 1 × PCR buffer (Fermentas), 0.2 mM deoxynucleoside triphosphates, 3.75 mM MgCl₂, 4% (vol/vol) bovine serum albumin (BSA, Sigma), 0.1 μM primers synthesized by IBA, 1U Taq polymerase (Fermentas), and template DNA (ca. 10 ng). After 5 min of denaturation at 94 °C, 30 thermal cycles of 45 s at 94 °C, 45 s at 56 °C, and 1.5 min at 72 °C were carried out. A final extension step at 72 °C for 10 min was performed to finish the reaction. One μL of the product of the first PCR was used as the template for a second PCR with bacterial DGGE primers F984-GC (5′-GC-clamp-AAC GCG AAG AAC CTT AC -3′) and R1378 (5′-CGG TGT GTA CAA GGC CCG GGA ACG-3′) according to Heuer et al. (1997).[64] The reaction mixtures (25 μl) consisted of 1 μL template, 1 × PCR buffer (Fermentas), 0.2 mM deoxynucleoside triphosphates, 2.5 mM MgCl₂, 4% (vol/vol) acetamid (Fluka), 0.1 μM primers and 1 U Taq DNA polymerase (Fermentas). PCR products were checked using standard agarose gel electrophoresis and ethidium bromide staining.[65] After amplification, 5 μL of the PCR product was subjected to electrophoresis on a 1.5% (w/v) agarose gel stained with ethidium bromide (0.01% v/v). Bacterial community fingerprints were generated using a CBS Scientific DGGE System (CBS Scientific Company, Del Mar, CA, USA). Approximately equal amounts of PCR products were loaded onto 6–9% polyacrylamide gel in 1x TAE buffer (20 mM Tris, 10 nM acetate, 0.5 mM EDTA, pH 7.4). The 6–9% polyacrylamide gel (bisacrylamide:acrylamide = 37.5 : 1) was made with a denaturing gradient ranging from 32 to 60%. Electrophoresis was performed at 60 °C for 16 h at 150 V. Following electrophoresis, the gels were silver stained. The solutions used were 0.1% (v/v) ethanol plus 0.005% acetic acid for fixation, 0.3 g silver nitrate for staining, freshly prepared developing solution containing 0.003% (v/v) formaldehyde and 0.33% NaOH (9%), and finally 0.75% sodium carbonate solution to stop the development. Gel images were acquired using a Molecular Image FX apparatus (Bio-Rad). The digitalized gels were analyzed with the software package Gelcompar 4.0 program (Applied Maths) as previously described by Smalla et al. (2001).[66]

After automatic band search, the bands detected were carefully checked and artefacts were removed. The band positions and their corresponding intensities (surface) from each treatment were exported to Excel (Microsoft). The band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane. The effect of each treatment was evaluated based on the calculated Bray–Curtis similarities, using analysis of similarities (ANOSIM) to assess the significance of separation between microbial communities from different treatments.[67] The R value in ANOSIM ranges from 0 to 1, where $R > 0.75$ indicates significant differences, $R > 0.5$ moderate separation and $R < 0.25$ high similarity.[68]

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