**Universidade de Aveiro** Departamento de Biologia
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# Porfirinas catiónicas na inactivação de bactérias patogénicas de peixes

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# Inactivation of fish pathogenic bacteria by cationic porphyrin

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 Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro

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**palavras-chave**

terapia fotodinâmica, sistema de aquacultura, porfirina catiónica, bacterias patogénicas de peixes, bactérias cultiváveis, estrutura da comunidade bacteriana

**resumo**

A importância da aquacultura tem vindo a aumentar de modo a compensar a progressiva redução mundial de peixes em ambiente natural. No entanto, esta indústria sofre frequentemente de elevadas perdas financeiras resultantes do desenvolvimento de infecções nos peixes causadas por microrganismos patogénicos, incluindo bactérias resistentes a antibióticos. Isto gera a necessidade de criar estratégias de controlo de infecções microbianas nos peixes que sejam benéficas para o ambiente, de modo a controlar a tornar a indústria e produção de peixe em aquacultura mais sustentável.

A terapia fotodinâmica antimicrobiana tem surgido como um método alternativo para o tratamento de doenças e para evitar o desenvolvimento da resistência aos antibióticos por parte das bactérias patogénicas. Esta terapia consiste na utilização de um fotossensibilizador que absorve luz visível, na presença de oxigénio, levando à formação de espécies altamente citotóxicas que inactivam os microrganismos. O objectivo deste trabalho foi investigar o efeito fotodinâmico de bactérias Gram (-) (*Vibrio anguillarum Vibrio cholerae, Vibrio, Aeromonas salmonicida, Photobacterium damselae damselae, Photobacterium piscicida damselae, Escherichia coli* e *Pseudomonas sp*) e Gram (+) (*Enterococcus faecalis* e *Staphylococcus aureus*), isoladas de uma aquacultura da Ria de Aveiro (Portugal). Foi usada a porfirina catiónica livre Tri-$Py^{+}$-Me-PF à concentração de 5.0 mM, e irradiação por exposição a luz branca artificial (40 W $m^{-2}$) durante 270 minutos. O efeito da terapia fotodinâmica na estrutura da comunidade bacteriana total e na abundância de bactérias cultiváveis da aquacultura foi também avaliado O impacto do tratamento fotodinâmico na abundância dos isolados bacterianos e na comunidade bacteriana cultivável da aquacultura foi avaliado através do número de unidades formadoras de colónias (UFC) após sementeira em placas de agar. Os efeitos ao nível da estrutura da comunidade bacteriana foram avaliados por "denaturing gel gradient electrophoresis (DGGE)". A exposição ao derivado porfirínico na presença de luz branca resultou numa diminuição de 7-8 log na abundância dos isolados bacterianos. A abundância de bactérias cultiváveis nas amostras de água provenientes do sistema de aquacultura foi também afectada, mostrando um decréscimo até 2 log na sobrevivência das células bacterianas. Porém, a taxa de inactivação variou significativamente nos diferentes períodos de amostragem. Os perfis de DGGE revelaram uma diminuição da diversidade da comunidade bacteriana total da água da aquacultura com a aplicação do tratamento fotodinâmico. Os resultados sugerem que a terapia fotodinâmica antimicrobiana pode ser considerada como um novo método para o controlo de infecções bacterianas em peixes em aquacultura, contudo como em regimes semi-intensivos e extensivos a comunidade bacteriana não patogénica pode ser afectada, antes da implementação da terapia fotodinâmica devendo ser feita uma avaliação rigorosa das implicações ao nível do ecossistema.

**keywords**

photodynamic therapy, aquaculture systems, cationic porphyrin, fish pathogenic bacteria , cultivable bacteria, bacterial community structure.

**abstract**

Aquaculture is assuming an increasing importance for the compensation of progressive worldwide reduction of natural fish stocks. Fish farming often suffer heavy financial losses resulting from fish infections caused by microbial pathogens, including multidrug resistant bacteria. Therefore, more environmentally-friendly 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. This therapy involves the use of a photosensitizer that absorbs visible light, in the presence of oxygen, leading to the formation of highly cytotoxic species that inactivate microorganisms. The aim of this work was to investigate the photodynamic inactivation of Gram (-) (*Vibrio anguillarum, Vibrio parahaemolyticus, Aeromonas salmonicida, Photobacterium damselae damselae, Photobacterium damselae piscicida, Escherichia coli and Pseudomonas* sp.) and of Gram (+) (*Enterococcus faecalis* and *Staphylococcus aureus*) bacteria isolated from an aquaculture system of Ria de Aveiro (Portugal). A free cationic porphyrin Tri-$Py^{+}$-Me-PF at 5.0 mM was used and the irradiation regime consisted of artificial white light (40 $W.m^{-2}$) for 270 minutes. The effect of the photodynamic process on the total bacterial community structure and on the abundance of cultivable bacteria from the aquaculture system was also evaluated. Photodynamic inactivation of bacterial isolates and cultivable bacteria was assessed by the number of colony forming units (CFU) in agar plates. The impact of photodynamic treatment in the total bacterial community structure of the aquaculture plant was evaluated by denaturing gel gradient electrophoresis (DGGE).The results showed that, in the presence of porphyrin derivative Tri-$Py^{+}$-Me-PF, the growth of bacterial isolates was inhibited, resulting in a decrease of ≈ 7-8 log after 270 minutes of irradiation. Total cultivable bacteria from the aquaculture were also considerably affected, showing decreases up to ≈ 2 log on cell survival by the photodynamic treatment. However, the inactivation rate varied significantly with the sampling period. DGGE profiles revealed a decrease in the diversity of the total bacterial community of the aquaculture water upon photodynamic treatment. Results indicate that photodynamic antimicrobial therapy can be regarded as new approach to control fish infections in aquaculture systems, but as non pathogenic microbial community of extensive and semi-intensive aquaculture systems can also be affected, a careful evaluation must be done before aPDT implementation in these systems.

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| | |
|---|---|
| *A. salmonicida* | *Aeromonas salmonicida* |
| aPDT | Antimicrobial photodynamic therapy |
| CFU | Colony forming units |
| DGGE | Denaturating gradient gel electrophoresis |
| *E. coli* | *Escherichia coli* |
| *E. faecalis* | *Enterococcus faecalis* |
| EDTA | Ethylenediaminetetraacetic acid |
| Gram (-) | Gram-negative |
| Gram (+) | Gram-positive |
| HPD | Hematoporphyrin derivative |
| LPS | Lipopolysaccharide |
| Mg2+ | Magnesium ions |
| $MgCl_2$ | Magnesium chloride |
| µL | Microlitre |
| µM | Micromolar |
| mL | Millilitre |
| nm | Nanometre |
| O2 | Molecular oxygen |
| PBS | Phosphate buffered saline |
| *P. damselae* subsp. *damselae* | *Photobacterium damselae* subsp. *damselae* |
| *P. damselae* subsp. *piscicida* | *Photobacterium damselae* subsp. *piscicida* |
| PACT | Photodynamic antimicrobial chemotherapy |
| PAR | Photosynthetically active radiation |
| PCR | Polymerase chain reaction |
| PDI | Photodynamic inactivation |
| PDT | Photodynamic therapy |
| PI | Photoinactivation |
| PS | Photosensitizer |
| $KCl_2$ | Potassium dichloride |

| | |
|---|---|
| Tri-Py+-Me-PF | 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide |
| *S. aureus* | *Staphylococcus aureus* |
| NaOH | Sodium hidroxide |
| TSA | Triptic soy agar |
| TSB | Triptic soy broth |
| *V. anguillarum* | *Vibrio anguillarum* |
| *V. parahaemolyticus* | *Vibrio parahaemolyticus*, |
| W | Watt |

# 1. Introduction

Aquaculture refers to the cultivation of aquatic plants and animals in controlled systems for commercial, recreational or resource management purposes in fresh, brackish and marine waters (Pillay, 2005).

Aquaculture provides nearly one-third of the world's seafood supplies and is one of the fastest growing agricultural sector. In Ria de Aveiro (Portuguese coast), fish intensive culture for human consumption, is an important economic activity. The growth and even survival of aquaculture industry is, however, threatened by uncontrolled bacterial diseases that cause extensive losses. The increasing problems with worldwide emergence of multidrug-resistant strains among common pathogenic bacteria and the concern about antibiotics spreading in the environment due to medical treatments in humans, in animal farms and fish farms, bring the need to find new methods to control fish pathogens (Schnick, 1988; Hoffman, 1974). To this end, photodynamic inactivation has been mentioned as a potentially viable alternative to antimicrobial drugs (Cassel, 2001).

## 1.1 Fish farming diseases

Cultured fish, animals and humans are constantly threatened by microbial attacks. The main biological agents that cause fish diseases are bacteria, viruses, parasites, oomycetes and, to a lesser extent, moulds (Shao, 2001; Olafsen, 2001; Toranzo et al., 2005; Almeida et al., 2009). Parasite-related food safety concerns in aquaculture are limited to a few helminthes species, and the hazards are largely focused on communities where consumption of raw or inadequately cooked fish is a cultural habit (WHO, 1995). Human viral diseases caused by the consumption of fish appear to present a low risk to human health, while viruses causing disease in fish are not pathogenic to man. Bacterial diseases are major problems in the expanding aquaculture industry (Sorimachi, 1985).

There are two broad groups of bacteria of public health significance that contaminate aquaculture products: those naturally present in the environment - indigenous microflora and those introduced through environmental contamination by domestic animals excreta and/or human wastes – non-indigenous microflora (Huss, 1994). Among these, Gram-negative [Gram (-)] are the major bacterial pathogens that affect various finfish (*Aeromonas salmonicida, Aeromonas hydrophila, Vibrio anguillarum, Edwardsielle*

*tarda, Pseudomonas sp., Yersinia ruckeri, Cytophaga columnaris, Flavobacterium sp., Photobacterium damselae* subsp. *piscicida (formerly Pasteurella), Photobacterium damselae* subsp. *damselae (formerly Vibrio damselae*) (Shao, 2001; Toranzo, Magariños et al., 2005; Meyer, 1991; Almeida et al., 2009; Olafsen, 2001) (Table 1). On the other hand, only few Gram-positive [Gram (+)] species affect finfish such as *Renibacterium salmoninarum, Nocardia spp., Mycobacterium sp., Streptococcus sp.* (Shao, 2001; Meyer, 1991; Toranzo et al., 2005). These pathogenic bacteria can cause several diseases; among the most important are septicemias, cutaneous lesions and skin destruction (Meyer, 1991).

**Table 1**: Pathogenic bacteria affecting marine fish cultures. (From Toranzo, 2005)

| Agent | Disease | Main marine hosts | Major serotypes/ serogroups | Vaccine availability | PCR-based diagnostic methods |
|---|---|---|---|---|---|
| Gram negative | | | | | |
| *Listonella anguillarum* (formerly *Vibrio anguillarum*) | Vibriosis | Salmonids, turbot, seabass, striped bass, eel, ayu, cod, red seabream | 3 | + | + |
| *Vibrio ordalii* | Vibriosis | Salmonids | 1 | − | − |
| *Vibrio salmonicida* | Vibriosis | Atlantic salmon, cod | 1 | + | − |
| *Vibrio vulnificus* | Vibriosis | Eels | 1 | + | + |
| *Moritella viscosa* (formerly *Vibrio viscosus*) | "Winter ulcer" | Atlantic salmon | 1 | + | − |
| *Photobacterium damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) | Photobacteriosis (Pasteurellosis) | Seabream, seabass, sole, striped bass, yellowtail | 1 | + | + |
| *Pasteurella skyensis* | Pasteurellosis | Atlantic salmon | ND | − | − |
| *Aeromonas salmonicida* subsp. *salmonicida* | Furunculosis | Salmonids turbot | 1 | (+)[a] | − |
| *Tenacibaculum maritimum* (formerly *Flexibacter maritimus*) | Flexibacteriosis | Turbot, salmonids, sole, seabass, gilthead seabream, red seabream, flounder | 2[b] | + | + |
| *Pseudomonas anguilliseptica* | Pseudomonadiasis "Winter disease" | Seabream, eel, turbot, ayu | 2 | (+)[c] | + |
| Gram positive | | | | | |
| *Lactococcus garvieae* (formerly *Enterococcus seriolicida*) | Streptococcosis or lactococcosis | Yellowtail, eel | 2 | (+)[d] | + |
| *Streptococcus iniae* | Streptococcosis | Yellowtail, flounder, seabass, barramundi | 2 | (+)[d] | + |
| *Streptococcus parauberis* | Streptococcosis | Turbot | 1 | + | + |
| *Streptococcus phocae* | Streptococcosis | Atlantic salmon | ND | − | − |
| *Renibacterium salmoninarum* | BKD | Salmonids | 1 | + | + |
| *Mycobacterium marinum* | Mycobacteriosis | Seabass, turbot, Atlantic salmon | ND[e] | − | − |
| *Piscirickettsia salmonis* | Piscirickettsiosis | Salmonids | 1 | (+)[f] | + |

[a] Limited protection in turbot.
[b] Further studies are needed to clarify the serotyping scheme.
[c] Under development.
[d] High protection but the duration is dependent on the fish host.
[e] No data reported.
[f] Questioned efficacy under field conditions.

Two important diseases of marine and estuarine fish and occasionally of freshwater fish are Vibriosis and Photobacteriosis. Species from the genera *Vibrio* (*Vibrio anguillarum, V. ordalii, V. salmonicida, V. vulnificus and V. parahemolyticus)* and *Photobacterium (Photobacterium damselae* subsp. *damselae)* cause Vibriosis. The symptoms of this disease are lethargy, slow growth, slow metamorphosis, tissue and appendage necrosis, muscle opacity (Defoirdt et al., 2007; Moriarty, 1997), septicemia with hemorrhage on the base of fins, exophthalmia and corneal opacity (Toranzo et al., 2005). Photobacteriosis is caused by the halophilic bacterium *Photobacterium damselae* subsp. *piscicida* which is highly pathogenic and affect a wide variety of fish species (Almeida et al., 2009; Toranzo et al., 2005), causing high levels of mortality reaching up to 100% in many aquacultures. The major symptoms of the bacterial infectious disease include presence of white nodules in the internal viscera, spleen and kidney. At water temperature between 18-20°C these bacteria cause high mortality of fish (Toranzo et al., 2005). The susceptibility to this disease is related to the age, structure and size of the fish as well as adverse environmental conditions (Toranzo et al., 2005). *Aeromonas salmonicida* affects many finfish, causing furunculosis, a severe septicemia disease in fish (Shao, 2001). This disease mostly affects a wide variety of salmonids in fresh and marine water, but also non-salmonid fish (Toranzo et al., 2005, Almeida et al., 2009). The disease is influenced by many factors such as high temperatures above 15°C, environmental stress, host-related factors and development of furunculosis (Toranzo et al., 2005). The major symptoms of the disease include deep ulcerative lesions, hemorrhages at the base of the fins around the gills and in the abdominal cavity, and reduction of appetite (Toranzo et al., 2005; Shao, 2001). Pseudomonadiasis is a fish disease caused by different species from genus *Pseudomonas* (*P. chlororaphis, P. anguilli septica, P. fluorescens, P. putida, P. plecoglossicida*) recognized as important pathogens of freshwater fish (Toranzo et al., 2005, Toranzo and Barja, 1993; Austin and Austin, 1999). This disease occurs especially in the winter months when temperature of water reaches values below 16°C. The main symptoms on the fish are abdominal distension, and hemorrhagic petechia in the skin and internal organs (Toranzo et al., 2005, Berthe et al., 1995; Doménech et al., 1997). Streptococcosis affects a variety of wild and cultured fish throughout the world (Toranzo et al., 2005; Romalde and Toranzo, 1999). This fish disease should be regarded as a complex of similar diseases caused by different genera and species capable of inducing a central

nervos damage characterized by suppurative exophthalmia and meningoencephalitis. There are cases of mortality caused by streptococcosis at temperatures around 15°C (Toranzo et al., 2005). Suppurative exophthalmia and meningoencephalitis are the characteristic symptoms of the fish affected by streptococcosis. Skin and gill infections are frequently caused by the *Cytophaga marina* and *Cytophaga columnaris*. These fish pathogenic bacteria occur in red and black sea breams, yellowtail and in flounder and cause respiratory distress of salmonids (Kusuda and Kimura, 1982; Toranzo et al., 2005; Wakabayashi et al., 1984).

Gram (+) *Renibacterium salmoninarum* causes a chronic systemic disease of salmonids and mortality in fish farming in fresh and marine environment. This bacterial kidney disease (BKD) causes exophthalmia, abdominal distension, pethechial hemorrhage and damage to the kidney and viscera on fish (Sanders and Fryer, 1980; Shao 2001, Toranzo et al., 2005). *Edwardsiella tarda* is another pathogenic bacterium that causes mortality on flounder (Nakatsugawa, 1983; Mekuchi, 1995). The Gram (+) *Yersinia ruckeri* causes enteric redmouth disease is also important pathogen of freshwater fish (Meyer, 2001; Post, 1983; Roberts, 1982).

The second most important class of pathogens in the aquaculture industry is the viruses that affect several fish (Munn, 2006; Shao, 2001) (Table 2). Viral diseases can become a serious problem for the production and health of fish in aquaculture (Meyer, 2001; Almeida et al., 2009). Some of these are already known, including Irodovirus, Rhabdovirus, Birnavirus, Nodavirus, Reovirus and Herpesvirus (Muroga, 2001; Suttle, 2007). Viral diseases can cause about 90% of mortality in fish (Meyer, 2001). However, viruses that cause disease in fish are not pathogenic for humans (Sorimachi, 1985). Viral diseases affect mostly trouts and salmons showing symptoms of infectious hematopoietic necrosis, infectious pancreatic necrosis and viral hemorrhagic septicemia (Almeida et al., 2009; Meyer 2001).

**Table 2**: Fish viral diseases in aquaculture systems (From Shao, 2001).

| Disease | Susceptible fish | Age of susceptibility | Usual temperature susceptibility range | Geographical distribution |
|---|---|---|---|---|
| Channel catfish virus (CCV) | Channel catfish | Fry and fingerlings | > 25°C | Southern USA |
| Infectious hematopoietic necrosis (IHN) | Salmonids | Mostly < 1 year old | < 18°C | Japan, N. America |
| Infectious pancreatic necrosis (IPN) | Salmonids | Fry and up to 20 weeks | Any | Worldwide |
| Spring viremia of carp (SVC) | Common carp | All but mostly yearlings | < 22°C | Europe |
| Viral hemorrhagic septicemia (VHS) | Salmonids (grayling, trout, whitefish) and pike | All | < 14°C | Europe (mainland) |

Oomycetes infectious diseases are mostly caused by members of *Saprolegnia*, affecting stressed fish that grow in unfavorable environmental conditions (Khoo, 2000). *Saprolegnia* infections are also a concern because they often cause secondary infections on fish infected by bacteria and viruses (Khoo, 2000). Infections caused by *Saprolegnia* are a major concern to a number of freshwater fishes (Leaño et al., 1999). Each freshwater fish is susceptible to, at least, one species of this group (Khoo 2000). Saprolegniosis present as symptoms white growth on the skin, gills or on fish eggs. Mycelium can to entrap sediment particles or algae, so the color of these lesions may appear to be red, brown or green. The gills into the pharynx, esophagus, liver, spleen, kidney, eye and brain can be infected by *Saprolegnia* (Khoo, 2000). The fishes most affected by *Saprolegnia* are trout and salmon (Meyer 2001). *Achyla* and *Dictyuchus* oomycetes also cause saprolegniosis in fish (Khoo, 2000; Hawke and Khoo, 2004). *Aphanomyces invadans* is also known as an oomycete pathogen of both estuarine and freshwater fish. The major symptoms caused by this pathogenic are granulomatous mycosis, epizootic ulcerative syndrome, red sore disease, and ulcerative mycosis (Willoughby et al., 1995; Khoo, 2000; Hawke and Khoo, 2004). Another fungal disease of the gill that affects a wide variety of freshwater fish is branchiomycosis. *Branchiomyces sanguinis* and *Branchiomyces demigrans* are two species from the genus *Branchiomyces* and they cause acute respiratory infections that occur when

water temperatures reach values above 20°C. Mortality of infected fish can be as high as 50% (Khoo, 2000).

In aquaculture several parasites can act as hosts for transmission of several fish diseases (Olafsen, 2001). The parasites cause reduction of appetite, low growth, physical deformities and mortality (Meyer, 2001). Most parasites are cosmopolitan and affect a variety of fish. There are species that cause mortality in all stages of life as *Ichthyophthirius*, *Ichthyobodo* and *Chilodonella*. The culture of marine fish is also affected by Cryptokaryon (Meyer, 2001).

The emergence of fish pathogenic microorganisms in aquaculture system appears when environmental conditions are unfavorable. This means that some factors such as oscillations in temperature, in salinity and the food given to fish increases the risk of development of pathogenic microorganisms causing fish diseases. Moreover, a polluted environment increases the risk of infection (Almeida et al., 2009)

### 1.2 Control of fish infections

Although disease prevention is the referred approach and the most cost-effective in the aquaculture industry, control of infections is difficult in fish farming conditions (Defoirdt et al., 2007). Poor water quality, ubiquitous nature and rapid spreading of pathogens, environmental adverse conditions, high stocking densities, different stages of the fish life cycle, resistance gains common pathogenic bacteria, low activity of chemotherapeutic agents against endospores and zoospores, and few drugs licensed for fisheries use are factors that difficult diseases prevention in aquaculture (Almeida et al., 2009).

Water treatment, incorporation in feed and injection are the primary means of administration of pharmaceutical agents. Adequate farming practices are critical components to control infectious diseases (Shao 2001).

### 1.2.1 Vaccination

Vaccination is the best method to prevent spreading of diseases among fish in aquaculture system (Defoirdt et al., 2007). Although the availability of vaccines for use in aquaculture is limited, photobacteriosis and vibriosis have been controlled through vaccination. Other bacterial diseases of farmed fish have been attempted with some success through vaccination as those transmitted by *Aeromonas salmonicida* and *Yersinia ruckeri* (Press and Lillehaug, 1995). It has been observed that vaccinated fish show greater growth and higher rates of survival compared to those who are not vaccinated. There is not yet a vaccine for bacterial kidney disease and rickettsial septicemia (Almeida et al., 2009). Due to the fact that diseases occur primarily at the fish fry age and as it is difficult to inject these small animals and also because fry age fish do not develop specific immunity, the application and development of vaccine is yet a concern in the field of aquaculture (Vadstein, 1997). The development of vaccines for fish viral diseases is still less improved. There are vaccines for infectious pancreatic necrosis viruses and infectious hematopoietic necrosis viruses (Christie, 1997). As for bacteria, it is difficult to vaccinate fry age fish that are also most susceptible to viral diseases (Fijan 1988; Leong, 1988).

### 1.2.2 Chemotherapy

Although the utilization of antimicrobial drugs has been a rapid and effective method to treat and prevent bacterial infections, the regular use of antibiotics has allowed the development of drug-resistant strains of bacteria. This problem may become serious because few drugs are licensed for fisheries use (Muroga, 2001; Regulamento CEE do Conselho nº 2377190). Different antibiotics have been used, including chloramphenicol, gentamycin, trimethoprim, tiamulin, tetracyclines, quinolones and sulfonamides (Table 3) (Defoirdt et al., 2007). The frequent use of artificial feeds supplemented with antibiotics in aquaculture to prevent diseases and to promote fish growth resulted in the increase of drug-resistant pathogenic bacteria that are turning antibiotics ineffective (Defoirdt et al., 2007). Formaldehyde, formalin formulations, malachite green, chlorine and hydrogen peroxide are also extensively used to control bacterial infections. As horizontal transfer of bacterial

genes in aquatic systems is high, the spread of antibiotic resistance in aquaculture constitutes an important threat to public health (Cabello, 2006). However, the biocides mentioned above are environmentally persistent, they produce a wide range of acute toxic effects on fish, causing carcinogenic and mutagenic risk to mammals, and affecting respiratory, immune and reproductive systems (Srivastava et al., 2004).

To reduce the risk of development and spreading of drug-resistant bacteria and to prevent microbial infections in aquaculture, other approaches environmentally more friendly must be developed. In line of this idea, the photodynamic therapy applied to aquaculture systems seems to be very promising.

**Table 3** : Drugs approved by the FDA for use in aquaculture (From Shao, 2001).

| Drug | Species | Indication |
|---|---|---|
| Oxytetracycline (Terramycin®) | Pacific salmon | Mark skeletal tissue |
| | Salmonids | Ulcer disease, furunculosis, bacterial hemorrhagic septicemia, and pseudomonas disease |
| | Catfish | Bacterial hemorrhagic septicemia and pseudomonas disease |
| | Lobster | Gaffkemia |
| Sulfadimethoxine, Ormetoprim (Romet®-30) | Salmonids | Furunculosis |
| | Catfish | Enteric septicemia |
| Tricanemethane-sulfonate (MS-222, TMS, Finquel®) | Fish and other aquatic poikilotherms | Sedation/anesthesia |
| Formalin (Paracid®-F, Formalin®-F, Parasite®-S) | Salmonids, catfish, largemouth bass, bluegill | Protozoa and monogenetic trematodes |
| | Salmodi and esocid eggs. | Fungi of the family Saprolegniaceae |
| | Penaeid shrimp | Protozoan parasites |
| Sulfamerazine | Rainbow, brook, and brown trout | Furunculosis |

## 2. Photodynamic therapy as an alternative to chemotherapy to inactivate microorganisms in the environment

### 2.1. Photodynamic therapy

More than 100 years ago, Raab (1900) reported the lethal effect of acridine orange and visible light on *Paramecium caudatum*, detecting the involvement of light and oxygen in the process. Later, von Tappeiner (1903) reported that the toxic effects observed in the presence of light were not due to heat. After further experiments in 1904, the direct influence of light was discarded and the term photodynamic effect was introduced (Raab, 1900; Tappeiner and Jesionek, 1903).

Nowadays, photodynamic therapy (PDT) has been successfully employed in medicine, namely in the treatment of many types of tumors, including skin cancer, oral cavity cancer, bronchial cancer, esophageal cancer, bladder cancer, head and neck tumors and in the treatment of some other non-malignant diseases in ophthalmology, dentistry (Foschi, et al. 2007; Souza, 2009) and dermatology (Kropf et al, 1998; James and Shinkai, 2002; Maiya, 2004; Komerik et al. 2003; Maisch et al., 2004; Maiya, 2004; Jori et al. 2006).

With the worldwide emergence of antibiotic resistance amongst pathogenic bacteria, a major research effort to find alternative antibacterial therapeutics has been done. Presently, antimicrobial photodynamic therapy (aPDT) is receiving considerable attention for its potentialities as a new form of antimicrobial treatment (Wainwright, 1998; Jori and Brown 2004; Jori et al., 2006; Maisch, 2009). It has been suggested that it is not probable that microorganisms can develop resistance to aPDT. Some authors used aPDT to inactivate drug-resistant bacteria and have found that these bacteria are equally as susceptible as their native counterparts to aPDT technology (Lauro et al 2002; Winckler, 2007; Jori, 2007; Pedigo et al., 2009). 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 (Dougherty et al. 1998; Wainwright 1998; Hamblin and Hasan, 2004).

Currently, the major use of aPDT is in the clinical area, in the disinfection of blood and blood derivates, oral cavity infections (such as periodontitis, endodontitis, treatment of superificial oropharyngeal *Candida* infections), *Tinea pedis* infection, and acne vulgaris (Wainwright, 1998; Bonnett, 2000; Wainwright, 2000; Jori et al. 2006; Maisch et al., 2007; Maisch, 2009). Recent studies have shown that photocatalytic therapy for microbial inactivation in drinking (Bonnett et al, 2006) and wastewaters (Jemli et al, 2002; Carvalho et al, 2007; Costa et al., 2008; Alves et al., 2009) is possible using solar irradiation.

Although just a few studies of aPDT have been done in aquaculture systems, preliminary results indicate that this technology has a high potential to disinfect aquaculture waters. The results of these studies show that Gram (-) and Gram (+) bacteria, yeasts, oomycetes and protozoa are effectively inactivated (5-6 logs decrease on cell survival) with micromolar doses of PS, after short periods of time (10 minutes) under low light intensities (50 mW $cm^{-2}$) in fish farming waters (Jori and Coppellotti, 2007). It was also showed that aPDT approach is effective to treat saprolegniosis of infected fish farming stocks (6-7 logs decrease on cell survival) without perilesional damage of the fish nor recurrence of the disease in infected sites or in other fish sites (Magaraggia et al., 2006). Wong et al (2005) showed also that *Vibrio vulnificus*, which frequently infects fish and contaminates fish farming waters is inactivated by aPDT.

The possibility of using PS immobilized in insoluble solid supports (Faust et al., 1999; Bonnett et al., 2006; Jiménez Hernández et al., 2006; Krouit et al., 2008; Almeida et al., 2009) allows the retention of the PS after photoinactivation (PI) process, avoiding its release to the environment, and, allowing its reuse making aPDT approach cost-effective and environmentally-friendly.

The effective inactivation of microorganisms without the formation of toxic by-products, the improbable development of photo-resistant strains associated to the possibility to irradiation fish farming waters with immobilized PS using solar radiation, suggest that aPDT can be considered an alternative technology to disinfect aquaculture waters.

## 2.2 Photosensitizers

Photosensitizers are natural or synthetic molecules that have good absorption capacity after irradiation. They include organic dyes (e.g., eosin, rose Bengal, acridine orange, methylene blue), porphyrins (Figure 1), phtahlocyanines and related tetrapyrrolic macrocycles (Merchat et al., 1996; Minnock et al., 1996; Wainwright, 1998, Wainwright, 2000, Jemli et al., 2002). The selection of the PS depends on the purpose of aPDT, but porphyrins and analogue derivatives have been the most promising compounds used in photochemotherapy.

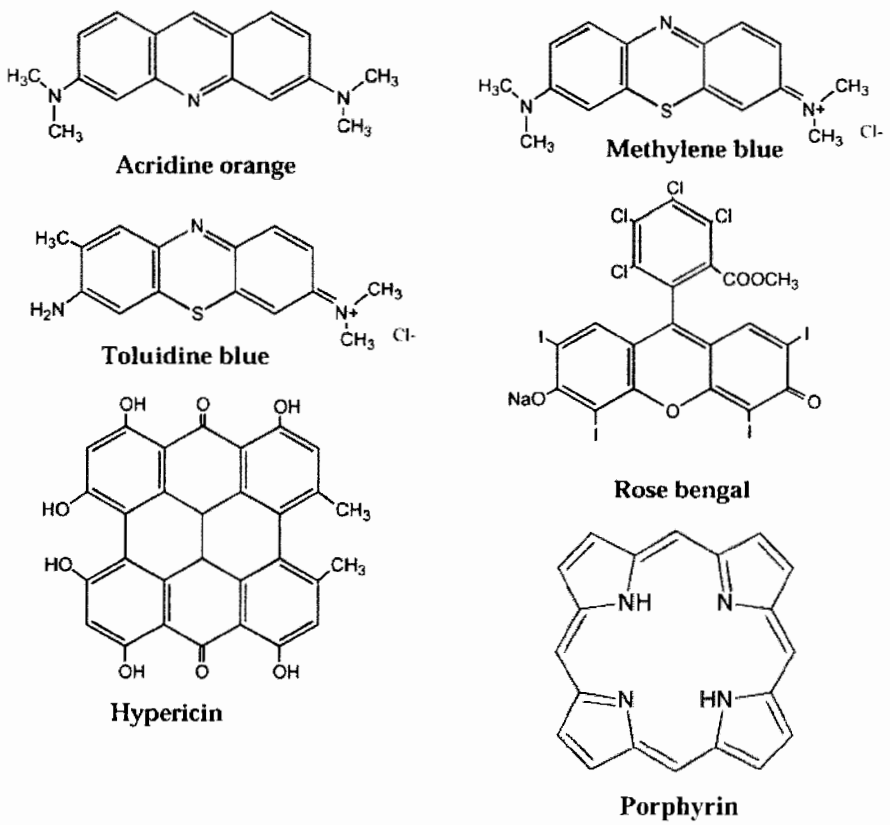



**Figure 1.** Chemical structures of some photosensitizers used for aPDT (From Hamblin, 2004).

Among the PS, porphyrins as haematoporphyrin derivative and its commercial version, Photofrin®, have been extensively studied and used for PDT treatment in several medical fields. Chlorine and benzoporphyrin derivatives have been developed and are now

under clinical trial (Carre, 1999). Various classes of chemical compounds, including phenothiazines, phthalocyanines, and porphyrins (Figure 2) have been successfully tested as photoinactivating agents against Gram (+), Gram (-) bacteria and another pathogenic microorganisms (Hamblin and Hasan 2004; Caminos et al., 2005; Demidova, 2005; Caminos and Durantini, 2006; Jori et al., 2006; Jori et al., 2007; Maisch, 2007).

Porphyrins are the photosensitizing agents that have been more frequently used in aPDT in environmental studies. They exert their biological action namely via the generation of singlet oxygen (Alouini, 2001; Magaraggia et al., 2006). They are many important biological representatives in nature, including hemes, chlorophylls, myoglobins, cytochromes, catalases, peroxidases, Vitamin B12 (Kristian, 2007).

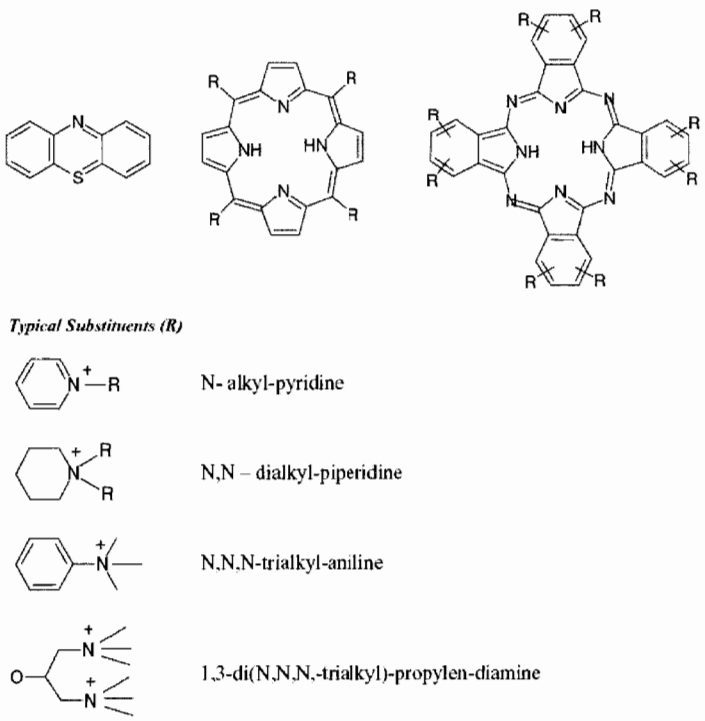



**Figure 2.** Basic chemical structure of phenothiazine, porphyrin, and phthalocyanine photosensitizers, and typical peripheral substituents (R) giving the photosensitizer a cationic character and enhancing the antimicrobial photosensitising efficiency (From Jori et al., 2006).

In PDT two oxidative mechanisms can occur after photoactivation of the photosensitizer named type I and type II pathways (Ochsner, 1997). The type I pathway involves electron or hydrogen atom transfer reactions from the PS triplet state, producing

radical forms of the PS or the substrate. These intermediates may react with oxygen to form cytotoxic species such as peroxides, superoxide ions, and hydroxyl radicals, which initiate free radical chain reactions (Athar et al. 1988). The type II pathway involves energy transfer from the PS triplet state to molecular oxygen to produce excited-state singlet oxygen ($^1O_2$) (De Rosa and Bentley, 2002) (Figure 3). The highly reactive singlet oxygen can oxidize many biological molecules, such as proteins, nucleic acids and lipids, and lead to cytotoxicity (Luksiene, 2003).

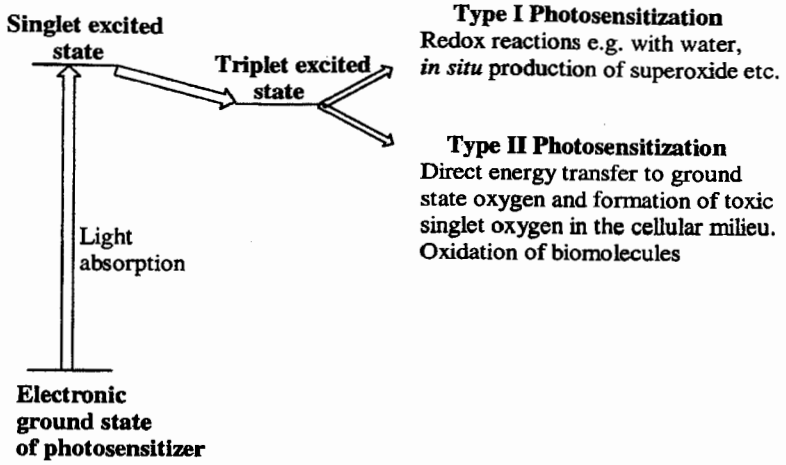



**Figure 3.** Mechanism of photosensitizer action (From Wainwright, 2000).

### 2.3. Photodynamic inactivation of bacterial cells

The mechanism of bacterial inactivation has not yet been elucidated. Over time many studies have shown that bacteria have been killed and inactivated by various combinations of PS and light. In the 90s, it was observed that there are fundamental differences in susceptibility to aPDT between Gram (+) and Gram (-) bacteria (Hamblin and Hasan 2004, Jori et al., 2004, Demidova and Hamblin, 2005; Banfi et al., 2006; Caminos and Durantini, 2006). Structural differences of PS show a degree of variation in bactericidal efficiency according to the organization of the cell wall (Figure 4). Many studies showed that Gram (+) bacteria are efficiently photoinactivated by a variety of sensitizers, whereas Gram (-) bacteria are usually resistant to the action of negatively

charged or neutral agents (Merchat and Bertolini et al., 1996; Merchat et al., 1996; Nitzan et al., 1998). The resistance of Gram (-) bacteria to the action of photoactivated sensitizers has been ascribed to the presence of highly organized outer membrane (including two lipid bilayers), which hinders the interaction of the photosensitizer with the cytoplasmic membrane and intercepts the photogenerated reactive species. (Merchat et al.,1996; Lazzeri et al., 2004).

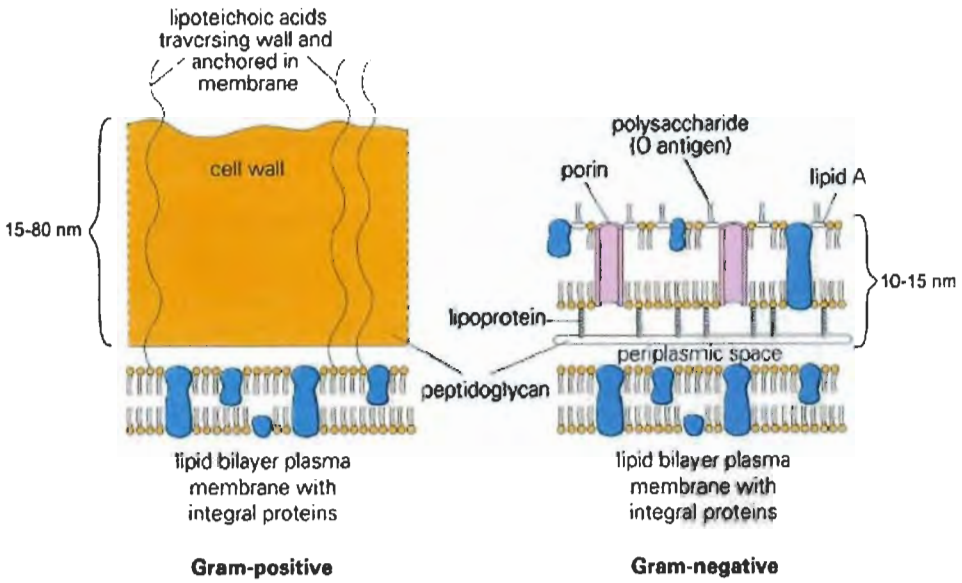



**Figure 4.** Schematic representation of the outer wall and cytoplasmic membrane structure in Gram (+) and Gram (-) bacteria (From Jori et al, 2006).

In Gram (-) bacteria, the lipopolysaccharide layer (LPS) offers some protection from the toxic effects of exogenous agents. This capacity enables these bacteria to survive in what otherwise must be considered hostile environments (Sanderson et al., 1974). The LPS has been shown to present a physical or chemical barrier through which free radicals and singlet oxygen generated outside of cells must pass to interact with a vital target, such as membrane or cytoplasmic components (Dahl et al., 1987). Gram (+) cells have only one lipid bilayer and a relatively permeable outer coat (Jori and Brown, 2004; Demidova and Hamblin, 2005).

Photodynamic inactivation of Gram (-) bacteria can be effective if the PS used has a cationic charge (Merchat and Bertolini et al., 1996; Merchat et al., 1996; Minnock et al., 1996) or if the bacterial membrane is permeabilized using polymyxin nonapeptid or

tris/EDTA with a non-cationic PS (Nitzan et al., 1992). These agents are able to disrupt the cell wall of the bacteria sufficiently to permit access of the PS which can then cause lethal damage to the cell when it is exposed to light (Malik et al., 1992). Therefore, singlet oxygen generated during the irradiation at the outer surface or in solution in close proximity to the cell is thought to diffuse into bacteria and produce fatal damage to lipids and proteins in the inner membrane (Dahl et al., 1987; Dahl et al., 1989). It has also been suggested that the outer membrane and LPS of Gram (-) bacteria represent vital targets for the lethal action of free radicals and singlet oxygen, since these can be removed without killing the cells (Dahl et al., 1989; Hamblin and Hasan, 2004).

It has been reported that cationic porphyrin derivatives are able to induce the photoinactivation of both Gram types (Hamblin et al., 2002; Jori and Brown, 2004). It has also been shown that the intracellular localization and binding site of the PS, which is highly affected by the structure and intramolecular charge distribution of it, is an important factor in anti-bacterial PDT (Merchat et al., 1996; Minnock et al., 1996). The positive charges promote an electrostatic binding of the porphyrin to the negatively charged sites at the outer membrane of Gram (-) bacteria, inducing damage that enhance the penetration of the PS (Merchat et al, 1996; Hamblin and Hasan, 2004).

Some studies compared the efficiency of synthetic cationic porphyrins with different charge distribution (tetra-, tri-, di- or monocationic), but the results are not consensual. Tetracationic porphyrins are efficient PS against both Gram types on visible light (Merchat et al., 1996). It was, however, also verified that some di- and tri- cationic porphyrins were more efficient than tetracationic ones, both against a Gram (+) and Gram (-) bacteria strains (Merchat et al., 1996) showing that a dicationic porphyrin as well as two tricationic porphyrins having a trifluoromethyl group were powerful photosensitizing agents against *Escherichia coli*.

Alves et al. (2009) concluded that there are some factors which increase the amphiphilic character of the porphyrins: the assymetric charge distribution at the peripheral position of the porphyrin, cationic charges combined in different patterns with highly lipophilic groups, introduction of aromatic hydrocarbon side groups and modulation of the number of positive charges on the PS. This increase in the amphiphilic character of the PS seems to enhance its affinity for bacteria which helps a better accumulation in the cells accompanied by an increase in the photocytotoxic activity (Alves et al., 2009).

### 2.4. Advantages of photodynamic therapy in the environment

According to Almeida et al., (2009), several aspects make aPDT an improved approach, relatively to use in the environment due to: (1) a broad spectrum of action of the PS, the ability of the PS to efficiently inactivate bacteria, viruses, fungi, and parasites in both the dormant and vegetative states contrarily to chemotherapy and phage therapy; (2) an efficient phototoxic activity against both wild and antibiotic-resistant microbial strains; (3) the lack of selection of photo-resistant microbial species; (4) a low mutagenic potential; (5) a high selectivity in the killing of pathogens as compared with the main constituents of potential host tissues; (6) a high selectivity in space and time, the microsecond short lifetime and high reactivity of singlet oxygen (the main pathway of aPDT inactivation), restricts the photooxidative damage to the microenvironment of the site where it is generated to about 0.1 μm; (7) the lack of generation of potentially dangerous or toxic by-products from photoinduced degradation of the photosensitizing agent; (8) the cost-effectiveness of the technology, as it is based on the use of visible light sources, as solar irradiation; (9) the possibility to reuse the immobilized PS which makes this technology less expensive and avoids its diffusion to the environment, preventing any risk of environmental contamination (Almeida et al., 2009).

## 3. Ecological impact of photodynamic therapy on general bacterial communities

Antimicrobial PDT apparently does not induce the selection of resistant microbial strains not the production of potentially dangerous or toxic by-products. However, as PS do not present microbial selectivity can, consequently, inactivate non-pathogenic bacteria that have an important ecological role in aquatic systems. So, before the application of aPDT in the environment, a careful evaluation must be done. As bacteria are the major player in the biogeochemical cycles in the aquatic environment they have an important ecological role in intensive and semi-intensive aquaculture system (Cho and Azam, 1988; Cho and Azam, 1990; Moriarty, 1997). Therefore, they represent a good choice to evaluate

the impact of aPDT. As only a small fraction of bacteria in the aquatic system is active, the evaluation of the total number of bacteria (active and inactive bacteria) is not a good option to evaluate the impact of this approach. The fraction of cultivable bacteria corresponds only to active bacteria and so, it is an appropriate indicator to evaluate the impact of the photochemical process in the aquaculture systems. However, as a big fraction of bacteria is non cultivable, 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 general bacterial community. DGGE is a nucleic acid based (DNA or RNA) technique which can be used to profile and identify dominant members of the microbial community based on their genetic fingerprint method that separates polymerase chain reaction (PCR)-generated DNA products (Saiki et al., 1988). DGGE of PCR-amplified ribosomal DNA fragments has been used frequently in microbial ecology studies (Crump et al., 2003; Muyzer et al., 1993; Muyzer et al., 1995; Muyzer and Smalla, 1998) Within a short period of time, this method has attracted the attention of many environmental microbiologists, and the technique is now used in many laboratories (Muyzer and Smalla, 1998; Ercolini, 2004). The PCR of environmental DNA can generate templates of different DNA sequences that represent many of the dominant microbial organisms. However, since PCR products from a given reaction are of similar size, conventional separation by agarose gel electrophoresis results only in a single DNA band that is largely non-descriptive. DGGE overcome this limitation by separating PCR products based on sequence differences that result in differential denaturing characteristics of the DNA (Muyzer and Smalla, 1998). In DGGE, the PCR products are separated in a polyacrylamide gel with increasingly higher concentrations of chemical denaturant. Upon reaching a threshold denaturant concentration, the weaker melting domains of the double-stranded PCR product will begin to denature (Fisher and Lerman, 1983). As differing sequences of DNA will denature at different denaturant concentrations it will be obtained a pattern of bands. Fingerprints can be uploaded into databases in which fingerprint similarity can be assessed to determine microbial structure. The number of bands corresponds to the number of predominant members in the microbial community.

## 4. Objective

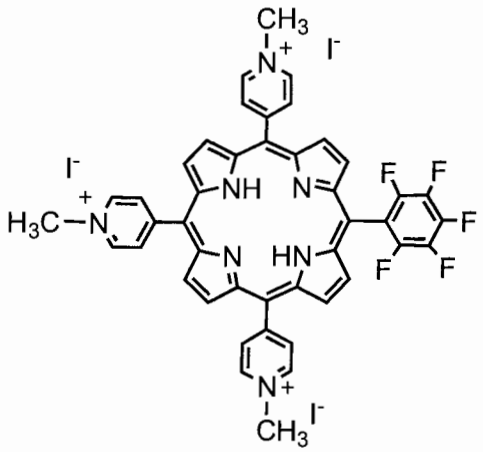
The aim of this work was to evaluate the applicability of aPDT to pathogenic fish bacteria. To reach this objective a cationic porphyrin derivative 5,10,15-tris(1-methylpyridinium-4-yl)-20 (pentafluorophenyl) porphyrin tri-iodide (Tri-$Py^{+}$-Me-PF), used as photosensitizer, was tested against nine 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*) isolated from a semi-intensive aquaculture system (Corte das Freiras) of Ria de Aveiro, in Portugal.

In order to evaluate the ecological impact of aPDT in the aquatic environment, the porphyrin effect was also tested directly on general bacterial community using the density of the cultivable bacterial fraction and the total bacterial community structure as indicators.

## 5. Materials and Methods

### 5.1 Photosensitizers

The porphyrin 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl) tri-iodide (Tri-Py$^{+}$-Me-PF) (Figure 5) used in this work was prepared in two steps. First, the neutral porphyrin was obtained from the Rothemund and crossed Rothemund reactions using pyrrole and the adequate benzaldehydes (pyridine-4-carbaldehyde and pentafluorophenylbenzaldehyde or 4-formylbenzoic acid) at reflux in acetic acid and nitrobenzene (Sirish, Chertkov et al. 2002; Tome, Neves et al. 2004). The resulting porphyrin was separated by column chromatography (silica) and then the pyridyl groups of porphyrin were quaternized by reaction with methyl iodide. 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. Porphyrin was provided by the Organic Chemistry group from the Department of Chemistry from University of Aveiro.




**Tri-Py$^{+}$-Me-PF**

**Figure 5.** Structure of the porphyrin derivative used for the photoinactivation of Gram (-) and Gram (+) bacteria.

## 5.2 Microorganisms and growth conditions

The photosensitizer was tested on isolated bacteria and on the bacterial community from the aquaculture water.

The bacteria strains used in this study were potential fish pathogenic bacteria previously isolated in our laboratory from the aquaculture systems (Carícias do Mar) of Ria de Aveiro, in Portugal. Seven Gram (-) strains (*Vibrio anguillarum*, *Vibrio parahaemolyticus*, *Aeromonas salmonicida*, *Photobacterium damselae* subsp. *damselae*, *Photobacterium damselae* subsp. *piscicida, Escherichia coli*, *Pseudomonas sp.*) and two Gram (+) strains (*Enterococcus faecalis* and *Staphylococcus aureus*) were used. The bacteria were stored at 4°C in tryptic soy agar (TSA, Merck). Before each assay the strains were grown aerobically for 24 hours at 26°C (*V. anguillarum*, *V. parahaemolyticus*, *A. salmonicida*, *Photobacterium dam.* subsp. *damselae*, *Photobacterium dam.* 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 26°C or 37°C (depending on the bacterial strain in study).

The water samples of aquaculture were collected two hours before low tide at 0.2 m from the surface in a culture tank of *Dicentrarchus labrax* and *Sparus aurata*. The water was collected with a sterilized bottle in different dates: 26$^{th}$ March 2009, 6$^{th}$ May 2009, 21$^{st}$ May 2009 and 26$^{th}$ June 2009. Water samples were protected from light and maintained at 18°C approximately until analysis.

## 5.3 Irradiation conditions

The effect of cationic porphyrin Tri-Py$^{+}$-Me-PF was evaluated by exposing the samples to white light in parallel (PAR radiation, 13 OSRAM 21 lamps of 18 W each one, 380-700 nm) with a fluence rate of 40 W m$^{-2}$, for 270 minutes, under 100 rpm stirring.

### 5.4 Experimental Setup

#### 5.4.1 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. Knowing that the inactivation of bacteria by cationic porphyrins is very sensitive to the ionic strength of the suspension medium (Lambrechts et al., 2004), 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 $\approx 10^{8}$ CFU mL$^{-1}$. In all experiments, 63 mL of bacterial suspension were aseptically distributed in 600 mL acid-washed and sterilized glass goblets (three goblets, 20 mL per each). The PS at 5.0 μM was added to one goblet (test goblet) and the other two goblets were used as dark and light controls. To the light control goblet no PS was added and the bacterial suspension was exposed to light. To the dark control goblet an appropriate volume of PS was added (to achieve a final concentration of 5.0 μM) and the goblet was protected from irradiation with aluminium foil. All goblets were subjected to an incubation period of 10 minutes under 100 rpm stirring at room temperature (20-25°C), prior to irradiation, to promote the porphyrin binding to bacterial cells. After this period, the test goblet and the light and dark controls were exposed to white light, at 20-25°C, during 270 minutes under stirring (100 rpm). A standard volume of 1 mL of test and control samples was collected at time 0 and after 15, 30, 60, 90, 180 and 270 minutes 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 hours of incubation at 26°C or 37°C, the number of colonies was counted on 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. The results are presented by survival curves plotted as logarithmic bacterial reduction (log CFU mL$^{-1}$) *versus* time of irradiation (in minutes).

### 5.4.2 Effect of aPDT on the cultivable bacterial density of aquaculture water

A water sample was collected from the aquaculture plant and was aseptically distributed in 600 ml acid-washed and sterilized glass goblets (15 mL per each of two goblets). The PS at 5.0 μM was added to one goblet (test goblet) and the other goblet was used as light control. The light control goblet was not added with porphyrin and was exposed to light. After the addition of the appropriate volume of porphyrin, 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: exposition of the test goblet and the light control to white light, at 20-25°C, under stirring (100 rpm); collection of 1 mL samples from each goblet at time 0 and after 60, 90 and 270 minutes of light exposure; serially dilutions and plating in duplicate in TSA medium. After 3-4 days of incubation at 25°C in the dark, the number of colonies was counted on the most convenient series of dilution. The results are presented by survival curves plotted as logarithmic bacterial reduction (log CFU $mL^{-1}$) *versus* time of irradiation (in minutes).

### 5.4.3 Effect of aPDT on the bacterial community structure

For the characterization of the bacterial community structure from the aquaculture plant, water samples were collected and aseptically distributed in 600 mL acid-washed and sterilized glass goblets (6 light control and 3 test goblet, 150 mL of water sample each). In the test goblets, an appropriate volume of Tri-$Py^{+}$-Me-PF was added to reach 5.0 μM of porphyrin derivative (final concentration). Light controls consisted of bacterial suspension only (no PS added). The test and light control goblets were exposed to white light (PAR radiation, 13 OSRAM 21 lamps of 18 W each one, 380-700 nm) with a fluence rate of 40 W $m^{-2}$), at 20-25°C, during 270 minutes under stirring (100 rpm). A 150 mL volume of test and control samples was collected at time 0 and after 270 minutes of light exposure and filtered through 0.2 μm polycarbonate filters (Poretics Products Livermore, USA). Collected cells were resuspended in 2 mL of TE buffer [10mM Tris HCl, 1mM ethylenediaminetetraacetic acid (EDTA), pH 8.0] and centrifuged. Lysozyme solution (2 mg $mL^{-1}$) was added to induce cell lysis, followed by incubation at 37°C for 1 h according

to the procedure described by Henriques et al (2004). DNA extraction was performed using the Genomic DNA Purification kit by MBI Fermentas (Vilnius, Lithuania). DNA was resuspended in TE buffer and stored at –20°C. The yield and quality of DNA were checked by electrophoresis on a 0.8% (w/v) agarose gel. PCR amplification of an approximately 400 bp 16S rDNA fragment (V6-V8) was performed using the primer set F968GC and R1401 (Nubel *et al.*, 1996). The reaction was carried in a Bio-Rad iCycler Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA) from MIDSCI. The PCR reaction mixture (25 µL) contained, approximately 50 to 100 ng of extracted DNA; 1x PCR buffer (PCR buffer without $MgCl_2$: PCR buffer with $KCl_2$, 1:1); 2.75 Mm $MgCl_2$; 0.2mM of each nucleotide; 0.1µM of each primer; and 1 U of Taq Polymerase (all reagents purchased from MBI Fermentas, Vilnius, Lithuania). Acetamide (50%, 0.5µl) was also added to the reaction mixture. The amplification protocol included a 4 minute initial denaturation at 94°C, 34 cycles of 95°C for 1 minute, 53°C for 1 minute and 72°C for 1 minute and 30 seconds, and a final extension for 7 minutes at 72°C. 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). DGGE was performed with the Dcode 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 hours at 150 V. Following electrophoresis, the gels were silver stained. After fixation with 0.1% (v/v) ethanol, 0.005% acetic acid for fixation, 0.3 g silver nitrate for 20 min, freshly prepared developing solution containing 0.003% (v/v) formaldehyde, 0.33% NaOH (9%) was added. The development was stopped using a0.75% sodium carbonate solution (Heuer et al., 2001). Gel images were acquired using a Molecular Image FX apparatus (Bio-Rad). The impact of the porphyrin effect on the diversity of bacterial community was assessed by determination of the number of bands in DGGE images in the samples and controls during exposure to white light for 270 minutes.

## 6. Results

### 6.1 Experiments with suspensions of isolated bacteria

According to results of the experiments, the porphyrin Tri-Py$^{+}$-Me-PF was effective against all the bacteria under study: Gram (-) strains (*V. anguillarum*, *V. parahaemolyticus*, *A. salmonicida*, *P. damselae* subsp. *damselae*, *P. damselae* subsp. *piscicida*, *E. coli*, *Pseudomonas sp.*) and Gram (+) strains (*S. aureus* and *E. faecalis* ), causing, in most cases, approximately 8 log reduction on cell survival, after 270 minutes of exposure to white light, with a fluence rate of 40 W m$^{-2}$. However, the PI process for Gram (+) bacteria was faster than for Gram (-) bacteria.

The results of light and dark controls showed no reduction on cell viability during all irradiation time when the bacterial suspension was exposed to light without PS and when the bacterial suspension is in contact with PS but protected from light. This indicates that the reduction on cell survival was due to the PI process.

As it can be observed, the major reduction (≈ 5,76 logs) on *V. anguillarum* viability was achieved after 60 minutes of irradiation. After 270 minutes of exposure to white light a reduction of ≈ 7.45 logs on cell viability was obtained (Figure 6A). On the other hand, ≈ 5.93 logs of reduction on *V. parahaemolyticus* viability was obtained after 30 minutes of irradiation and photoinactivation to the detection limit occurred after 270 minutes of irradiation causing ≈ 7.97 logs of reduction on cell viability (Figure 6B).

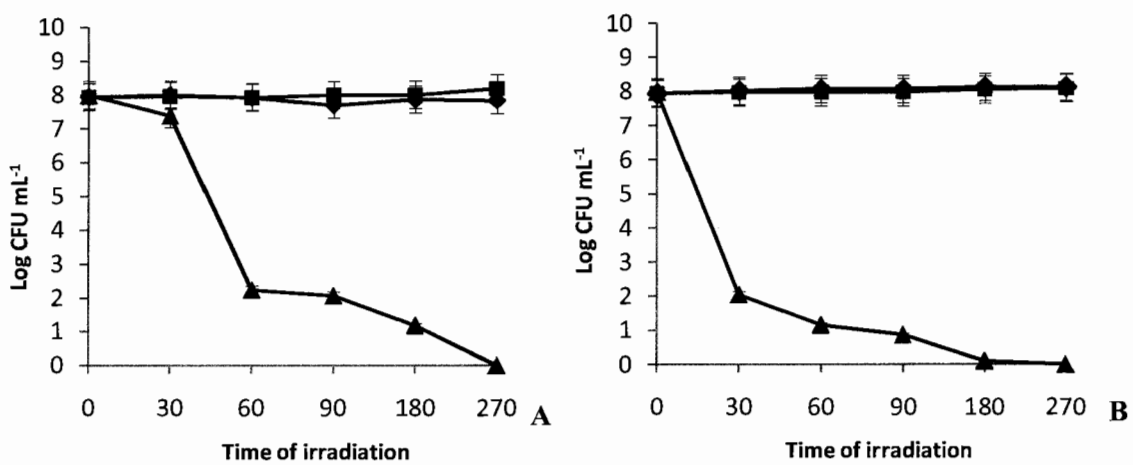



**Figure 6.** Variation on viability of *Vibrio anguillarum* (**A**) and *Vibrio parahaemolyticus* (**B**) isolated from aquaculture water in the presence of porphyrin Tri-Py$^{+}$-Me-PF at 5.0 μM (▲) after 30, 60, 90, 180 and 270 minutes of 40 W m$^{-2}$ irradiation. Light control (♦) had not added porphyrin and dark control (■) had added porphyrin at 5.0 μM.

The PI process was also effective against *P. damselae* subsp. *damselae* showing ≈ 7.91 logs decrease on cell viability after 90 minutes of irradiation (Figure 7A). However, the PI process for *P. damselae* subsp. *piscicida* was able to cause the same effect on cell viability (≈ 8.05 logs decrease) only after 180 minutes of irradiation (Figure 7B). The major reduction on cell viability was similar for these two strains, causing approximately 5.7 logs decrease, after 15 minutes of irradiation.

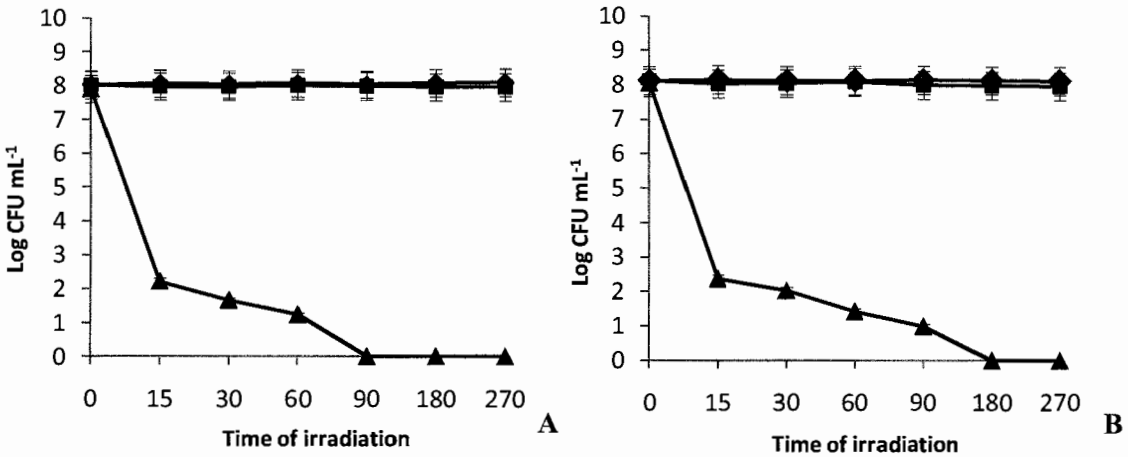



**Figure 7.** Variation on viability of *Photobacterium damselae* subsp. *damselae* (**A**) and *Photobacterium damselae* subsp. *piscicida* (**B**) isolated from aquaculture water in the presence of porphyrin Tri-Py$^{+}$-Me-PF at 5.0 μM (▲) after 15, 30, 60, 90, 180 and 270 minutes of 40 W m$^{-2}$ irradiation. Light control (♦) had not added porphyrin and dark control (■) had added porphyrin at 5.0 μM.

The results showed that the viability of *A. salmonicida* was also affected by the PI process with the cationic porphyrin but PI to the detection limit was not observed after 270 minutes of irradiation. However, reductions on cell survival of 5.28 logs and 7 logs were obtained after 30 and 270 minutes of irradiation, respectively (Figure 8).

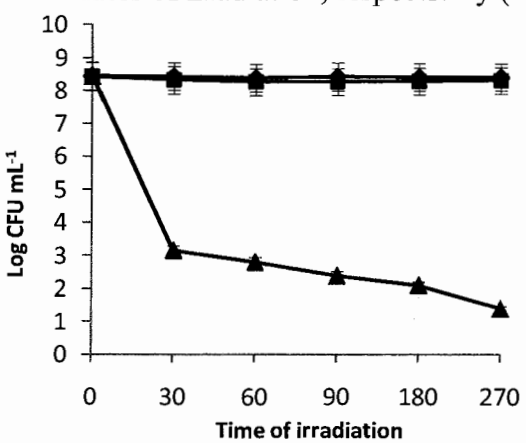



**Figure 8.** Variation on viability of *Aeromonas salmonicida* isolated from aquaculture water in the presence of porphyrin Tri-Py$^{+}$-Me-PF at 5.0 μM (▲) after 30, 60, 90, 180 and 270 minutes of 40 W m$^{-2}$ irradiation. Light control (♦) had not added porphyrin and dark control (■) had added porphyrin at 5.0 μM.

Tri-Py$^{+}$-Me-PF was also a good PS against *E. coli* and *Pseudomonas sp.*, affecting greatly cell survival. It was observed a reduction of 7.98 logs on *E.coli* viability after 180 minutes of irradiation (Figure 9A). On the other hand, a reduction of 7.97 logs on *Pseudomonas sp.* viability was obtained only after 270 minutes of irradiation (Figure 9B).

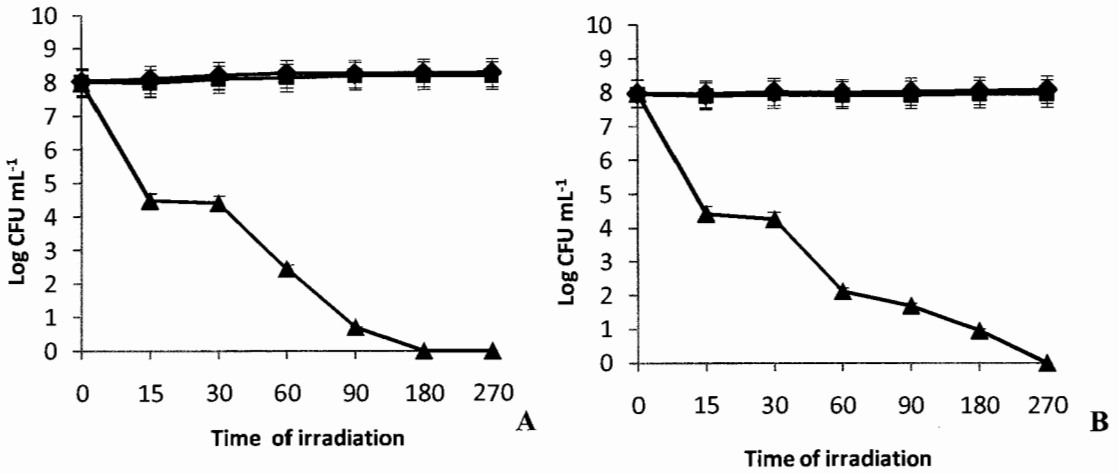



**Figure 9.** Variation on viability of *Escherichia coli* (**A**) and *Pseudomonas sp.* (**B**) isolated from aquaculture water in the presence of porphyrin Tri-Py$^{+}$-Me-PF at 5.0 μM (▲) after 15, 30, 60, 90, 180 and 270 minutes of 40 W m$^{-2}$ irradiation. Light control (♦) had not added porphyrin and dark control (■) had added porphyrin at 5.0 μM.

The PI process showed that Gram (+) strains are more easily photoinactivated than Gram (-) causing a rapid decrease on cell survival with the PS used. It was observed a 7.82 logs reduction on *E. faecalis* viability and PI to the detection limit after 90 minutes of irradiation (Figure 10A). Results for *S. aureus* showed that cell viability was also affected causing a reduction of 7.96 logs after 60 minutes of irradiation (Figure 10B). The major reduction on cell viability for both strains was obtained after 15 minutes of irradiation causing approximately 6 logs survivors reduction.

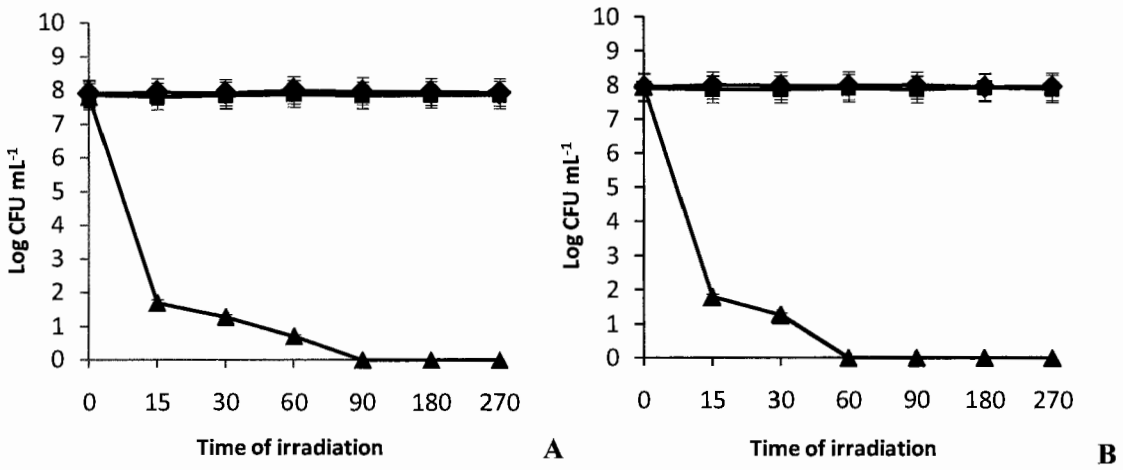



**Figure 10.** Variation on viability of *Enterococcus faecalis* (**A**) and *Staphylococcus aureus* (**B**) isolated from aquaculture water in the presence of porphyrin Tri-Py$^{+}$-Me-PF at 5.0 μM (▲) after 15, 30, 60, 90, 180 and 270 minutes of 40 W m$^{-2}$ irradiation. Light control (♦) had not added porphyrin and dark control (■) had added porphyrin at 5.0 μM.

### 6.2 Effect of aPDT on the cultivable bacterial density of aquaculture water

In this study, the results demonstrate that porphyrin Tri-Py$^{+}$-Me-PF was effective to inactivate cultivable bacteria that present a cell density of around 100 CFU per mL. However, the PI of cultivable bacterial by this porphyrin at 5.0 μM varied during the sampling period.

The results showed that the bacterial viability of samples A and D was affected by the PI process to the detection limit (2 log reduction) after 90 and 270 minutes of irradiation, respectively (Figure 11A and 11D). On the other hand, the PI process for samples B and C was least effective, showing a 1,25 log and 1,22 log reduction on cell viability, respectively, after 270 minutes of irradiation (Figure 11B and 11C). Light and dark controls did not show a significant reduction during the irradiation time (Figure 11), indicating that reduction on cell viability after irradiation of treated samples was due to the photodynamic effect of the PS.

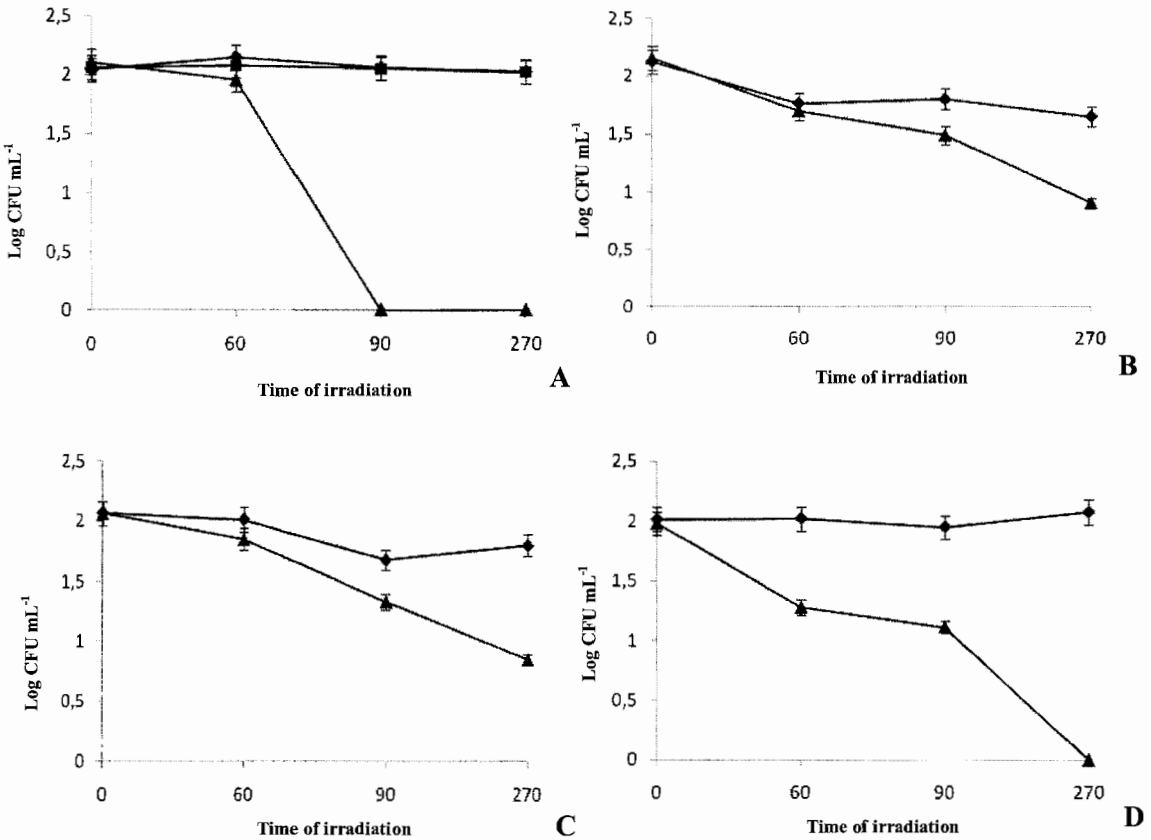



**Figure 11.** Logarithmic reduction of cultivable bacterial density in 4 different days: 26th March 2009 (**A**), 6th May 2009 (**B**), 21st May 2009 (**C**) and 26th June 2009 (**D**) with porphyrin Tri-Py$^{+}$-Me-PF at 5.0 μM (▲) after 60, 90 and 270 minutes of a 40 W m$^{-2}$ irradiation. Light control (♦) had not added porphyrin and dark control (■) had added porphyrin at 5.0 μM.

### 6.3 Effect of aPDT on the bacterial community structure

The effect of the photodynamic process on the total bacterial community structure of the aquaculture system was evaluated by DGGE after PCR-amplification of 16S rDNA gene fragments.

The DGGE profile resulting from separation of gene fragments of 16 S rDNA amplified by PCR is shown in figure 12.

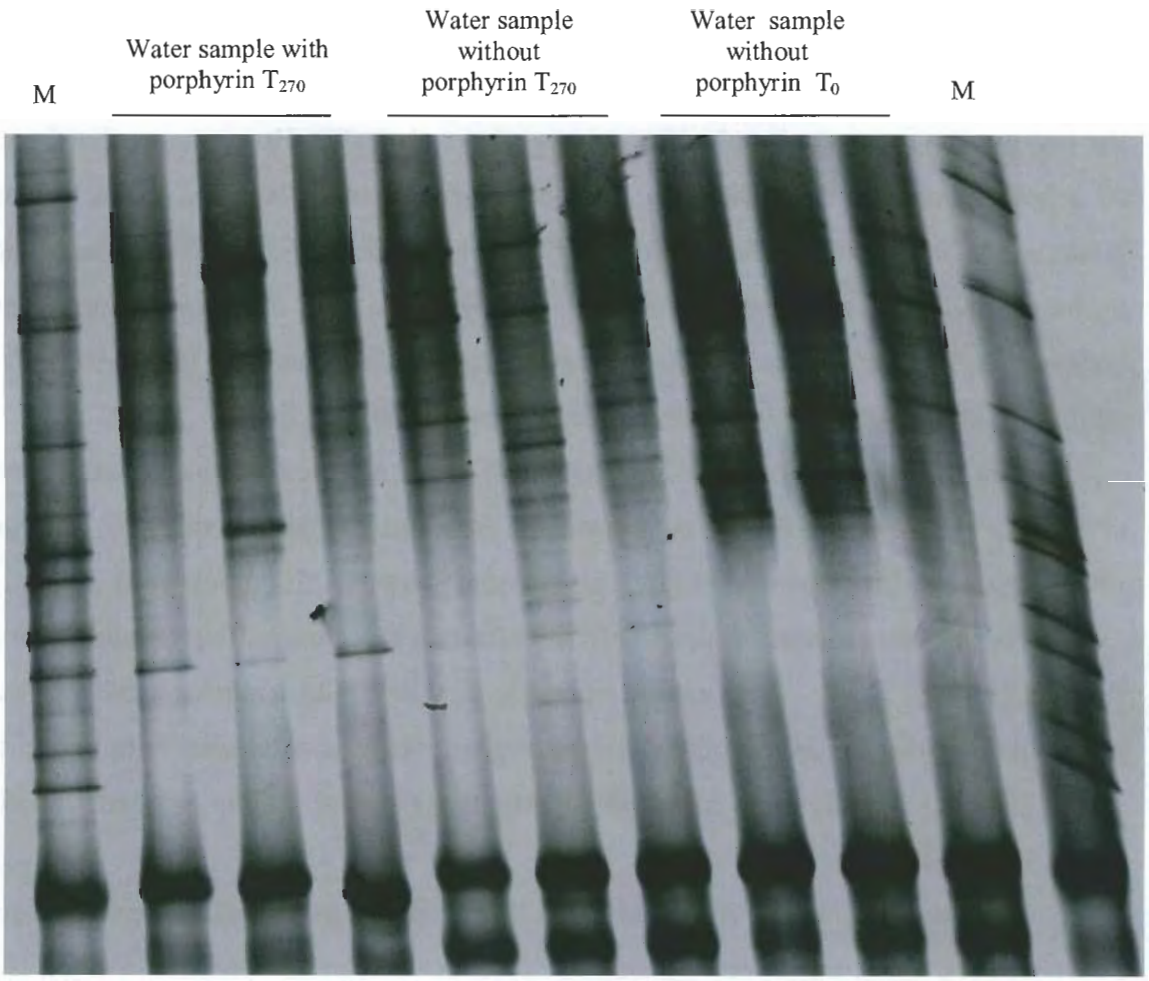



**Figure 12.** DGGE profile of 16S rDNA fragments after cationic porphyrin Tri-$Py^+$-Me-PF addition to bacterial community of an aquaculture system. M- molecular marker

DGGE profiles revealed a complex and specific pattern of bands (Figure 12) in the bacterial community of the aquaculture plant. Exposure of the water sample to white-light resulted in modifications in the bacterial community profile, comparatively to the original sample, revealed from differences in terms of relative intensity and band positioning between the two samples. In the presence of porphyrin, irradiation for 270 minutes led to a reduction in the number of bands detected in DGGE profiles. These results indicate that Tri-$Py^+$-Me-PF porphyrin at 5.0 μM caused a decrease on the diversity of the total bacterial community of the aquaculture water after 270 minutes of irradiation.

## 7. Discussion

Aquaculture provides nearly one-third of the world's seafood supplies and is one of the fastest growing agricultural sectors. (Almeida et al. 2009). However the development of infections caused by pathogenic microorganisms, namely bacteria, cause extensive losses in the aquaculture industry. Although the chemotherapy treatment has been shown to be a rapid and effective approach to prevent and treat infections, emergence of resistance to the more frequently chemotherapeutics creating a need for the development of alternative strategies to inactive microorganisms in fish farming plants.

Photodynamic antimicrobial therapy (aPDT) has emerged as an alternative method to inactive microorganisms and to prevent the development of infections by pathogenic microorganisms (Caminos et al. 2005; Caminos and Durantini, 2006; Jori et al. 2006).

The results obtained in this study indicate that the combination of porphyrin Tri-$Py^{+}$-Me-PF and visible light could represent a viable and environmentally friendly alternative for the control of potentially pathogenic bacteria in waters from aquaculture systems, as has already been investigated (Magaraggia et al. 2006, Jori and Capellotti, 2007).

However when aPDT is applied to the destruction of pathogenic microorganisms in extensive and semi-intensive aquaculture systems, a careful implementation of the procedure must be done, in order to avoid the inactivation of the non-pathogenic bacteria.

In this work, nine fish pathogenic bacteria isolated from the aquaculture systems studied were efficiently photoinactivated using a low concentration of porphyrin (5μM). In general, fish pathogenic bacteria were inactivated to the detection limits (≈8 logs after 270 minutes of irradiation). Nevertheless, the efficiency of the photoinactivation process varied among the bacterial strain. As previously reported (Hamblin and Hasan, 2004; Jori and Brown, 2004; Demidova and Hamblin, 2005; Banfi, Caruso et al. 2006; Caminos and Durantini, 2006), the Gram (+) bacteria were inactivated faster than Gram (-) bacteria, but for both groups of bacteria the photoinactivation varied with the bacterial strains, namely for Gram (-) group.

The photoinactivation for both Gram (+) bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) was similar, being both inactivated until the detection limits after 60-90 minutes of irradiation. The two strains of *Photobacterium* and *E. coli* were

inactivated to the detection limit after 90-180 minutes of irradiation. The two strains of *Vibrio* and *Pseudomonas* were totally inactivated after 270 minutes of irradiation. On the other hand, *A. salmonicida* was not completely inactivated during 270 minutes of exposure to the white light, after which around 1 log of cell survival to the photoinactivation process was observed. Although all Gram (-) bacteria have a thin layer of peptidoglycan and an outer membrane of lipopolysaccharide, the composition of the outer membrane varies among bacteria (Sanderson et al., 1974, Dahl et al., 1987, Jori and Brown, 2004; Demidova and Hamblin, 2005). The outer membrane acts as a physical and functional barrier between the cell and the outside, allowing the passage of nutrients and preserving the integrity of the cell. All it has been shown that PS do not need to penetrate into the cell to inactive microorganisms (Dahl et al., 1987, Dahl et al., 1989; Hamblin and Hasan, 2004), the structural role of the outer membrane in preserving the cell integrity seems to be the target of the photoinactivation process. The structure of the outer membrane of *A. salmonicida* seems to preserve more efficiently the cell than the structure of the outer Gram (-) bacteria. In fact, a few Gram (-) bacteria tested in this study were the most resistant to antibiotics (Pereira, personal communication).

It is important to note that in this study bacterial concentrations used were much higher than those found in aquatic system (6-7 logs higher) and that it was used a light intensity (40 $W.m^{-2}$) much lower than that observed in dark days of winter. Consequently, the application of aPDT to natural waters with low concentration of pathogenic bacteria and using sunlight as the light source (Alves et al., 2008) will probably be much more effective to inactive fish pathogenic bacteria.

In extensive and semi-intensive aquaculture systems non-pathogenic bacteria have a central role in the functioning and productivity of these ecosystems. Bacteria are the most important biological component involved in the turnover (transformation and remineralization) of organic matter in aquatic systems (Cho and Azam, 1990; Pomeroy et al, 1991). In coastal waters, heterotrophic bacteria may utilize as much as 40% of the carbon fixed by the primary producers (Cole et al. 1988; Cho and Azam, 1990; Dueklov and Carlson, 1992). Heterotrophic bacteria are particularly skilled for organic matter transformation. They hydrolize dissolved and particulate organic matter, can use substrates of difficult degradation and even different allochtonous compounds as further sources of organic carbon. They convert dissolved organic carbon that would inevitably be lost to

other higher trophic levels (microbial loop). Through remineralization of organic matter, bacteria regenerate nutrients which are then use by primary producers (Simon and Azam, 1989). Heterotrophic bacteria respire organic carbon to inorganic carbon at high rates, making bacterial respiration the major fraction of the total measure respiration in most aquatic systems (Williams, 1981), reaching values up to 40% of the total planktonic respiration (Cole and Pace, 1995).

As aPDT do not present selectivity relatively to bacteria, potentially inactivating all bacteria, a careful environmental evaluation must be done before the implementation of a procedure to photoinactive bacteria in aquaculture systems. The results of this study indicate that the cultivable fraction of the heterotrophic bacteria from the aquaculture plant studied, including pathogenic and non-pathogenic bacteria, was inactivated by the photodynamic process, but the efficacy of the inactivation varied during the sampling period. The cultivable bacteria in a concentration of around 100 CFU $m.L^{-1}$ were more efficiently inactivated in March and in June 2009 (2 log reduction on cell survival) relatively to May 2009 (1.2 log reduction on cell survival). A possible explanation to the observed of inactivation could be the variation of the environmental conditions. However, temperature, salinity, pH and dissolved oxygen were similar in the four sampling dates and laboratory experiments in which these parameters varied showed that photoinactivation was not affected (data not shown). However, the structure of bacterial community is affected by environmental conditions (Henriques et al., 2004) and a clear pattern of seasonal variation on the structure of bacterial communities has been observed (Fandino et al., 2001) in the water from aquaculture system even for pathogenic bacteria (data not shown). Consequently, the seasonal variation of photoinactivation observed for the cultivable bacteria can be due to differences in bacterial community structure. In fact, DGGE results showed that the members of the natural bacterial community of the aquaculture water are not similarly affected by the aPDT.

DGGE profiles showed that the bacterial community structure of the aquaculture system was affected by aPDT, but not all bacterial groups are inactivated. For water samples added of porphyrin and exposed to light during 270 minutes, a reduction on the number of bands relatively to the non added water samples was observed. This indicates that some bacterial groups were inactivated by the porphyrin. However, in these water samples bands not detected in the original water sample, appeared after exposition. This

indicates that some bacterial groups of aquaculture waters were not inactivated by aPDT. These groups of bacteria resistant to photoinactivation are not, however, dominant in terms of abundance, since they were not detected in non added samples. DGGE approach detects the most representative groups of bacteria. However, after photoinactivation, destruction of some representatives groups of bacteria allowed the detection of bacterial groups less significant in the original sample.

During the last two decades aPDT has been tested on a great number of bacteria, namely pathogenic bacteria and frequently on pathogenic multi-drug resistant bacteria. All the bacteria tested were inactivated by photoinactivation therapy. However, these studies only used cultivable bacteria, isolated from clinical samples and from the environment. Probably the bacteria resistant to aPDT correspond to non-cultivable groups. In fact, only a small fraction (less than 1%) of aquatic bacteria is cultivable (Amato et al., 2005). To a better evaluation of the efficiency of aPDT on aquaculture systems, the resistant bacteria must be identified by sequencing and by studying the effect of aPDT on bacterial community by quantitative PCR.

In conclusion aPDT is effective to inactive pathogenic bacteria of fish, being a promising alternative approach to traditional methods of desinfection. However, as aPDT is not selective for pathogenic microorganisms, the non pathogenic microbial community of extensive and semi-intensive aquaculture systems can also be affected. As non pathogenic bacteria have an important ecological role in the biogeochemical cycles in these aquacultures systems, a careful evaluation must be done before aPDT implementation in these systems.

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