Potential applications of porphyrins in photodynamic inactivation beyond the medical scope

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Abstract

Although the discovery of light-activated antimicrobial agents was reported in the 1900s, only more recently research work has been developed towards the use of photodynamic process as an alternative to more conventional methods of inactivation of micro(organisms). The photoprocess causes cell death through irreversible oxidative damage by reactive oxygen species produced by the interaction between a photosensitizing compound and a light source.

With great emphasis on the environmental area, photodynamic inactivation (PDI) has been tested in insect eradication and in water disinfection. Lately, other studies have been carried out concerning its possible use in aquaculture waters or to the control of food-borne pathogens. Other potential applications of PDI in household, industrial and hospital settings have been considered.

In the last decade, scientific research in this area has gained importance due to great developments in the field of materials chemistry but also because of the serious problem of the increasing number of bacterial species resistant to common antibiotics. In fact, the design of antimicrobial surfaces or self-cleaning materials is a very appealing idea from the economic, social and public health standpoints. Thus, PDI of micro(organisms) represents a promising alternative.

In this review, the efforts made in the last decade in the investigation of PDI of (micro)organisms with potential applications beyond the medical field will be discussed, focusing on porphyrins, immobilized or not on solid supports, as photosensitizing agents.

Keywords porphyrin; photosensitizer; photodynamic inactivation; disinfection; nanomaterials; self-cleaning materials

1. Introduction

Photodynamic therapy refers to the use of a light source (visible light of an appropriate wavelength), an oxidizing agent (molecular oxygen, O_2) and an intermediary agent (named photosensitizer, PS) able to absorb and transfer the energy of the light source to molecular oxygen leading to the formation of highly cytotoxic species (singlet oxygen [1O_2], hydrogen peroxide [H_2O_2], and/or free radicals, such as superoxide [$O_2^{\cdot-}$] and hydroxyl radical [$HO^{\cdot}$]), causing a multi-targeted damage and destruction of living tissues [1, 2]. The generation of these reactive oxygen species (ROS) can occur *via* two mechanisms or pathways, known as type I and type II, which require the presence of O_2 (Fig. 1). In the presence of light (**h** ν), the photosensitizer in the singlet ground state absorbs a photon, affording the excited singlet state. Then, it can lose energy by returning to the singlet ground state with fluorescence emission (**F**) or, through an intersystem crossing (**ISC**) process, it can be converted in the long-lived triplet state. This excited triplet-state PS can decay to ground state by phosphorescence emission (**P**) or can react with a substrate, namely an electron donor molecule. In this case the formation of radical ions can occur giving rise to radical ions which react with ground state oxygen (3O_2), originating ROS (type I mechanism). Alternatively, the excited triplet-state PS can transfer energy directly to molecular oxygen affording the excited singlet state (1O_2) (type II mechanism). Both photoprocesses may occur simultaneously but type II is, in general, the predominant one. The cytotoxic species can cause irreversible damage to proteins, nucleic acids and lipids [3, 4].

The advantage of being a process without a specific cell target renders photodynamic inactivation (PDI) effective in the oxidation of different biomolecules with the consequent destruction of several cell types. In fact, this methodology has a broad spectrum of activity and, using the same PS, is able to destroy human cells [1], viruses [5], bacteria [6], molds [7], yeasts [8], protozoa [9], helminths [10] and insects [11].

Fig. 1. Schematic representation of the photoprocesses that can occur during photodynamic inactivation. ISC, intersystem crossing; IC, internal conversion.

Moreover, the ability to structurally tailor the PS as well as to successfully link it to other molecules, with a high degree of specificity (e.g., antibodies, enzymes, nucleic acids), or to solid supports gives this therapy a multiplicity of clinical and non-clinical applications.

The discovery that positively charged PS could effectively inactivate Gram-negative bacteria without the addition of permeabilizing agents [12, 13] brought a new impetus to the investigation on the PDI of microorganisms as a new therapeutic modality.

The difference in susceptibility between the two types of bacteria, Gram-negative and Gram-positive, is explained on the basis of the structural features of their cell wall (Fig. 2). Gram-positive bacteria have a cell wall composed of lipoteichoic and teichoic acids organized in multiple layers of peptidoglycan, which confers a degree of porosity to bacteria so as to facilitate the anchoring and entry of PS into the cell [14, 15]. In Gram-negative bacteria, the presence of a complex outer membrane in the cell wall, consisting of phospholipids, lipopolysaccharides, lipoteichoic acids and lipoproteins creates an impermeable barrier to antimicrobial agents [14, 15]. The interaction between the cationic PS and the constituents of the Gram-negative cell wall generates electrostatic interactions that promote destabilization of the native organization of the wall, allowing the binding and eventual entry of the PS molecules into the cell [14, 15]. In the case of fungi, the cell wall contains chitin, glucans and lipoproteins that represent a barrier with intermediate permeability in comparison to Gram-positive and Gram-negative bacteria [16]. With regard to viruses, enveloped viruses are more easily inactivated than non-enveloped ones, but some studies show that non-enveloped viruses can also be efficiently inactivated by the phototoxic action of cationic PS [17], being the efficiency of their inactivation similar to that of Gram-negative bacteria [18].

Fig. 2. Schematic representation of the cellular envelope of Gram-positive (left) and Gram-negative bacteria (right).

In recent years, the synthesis of new compounds for PDI has grown dramatically, many of them with very good inactivation results. Several classes of PS, such as phenothiazinium dyes (methylene blue, toluidine blue O), naturally occurring PS (chlorophylls, psoralens, perylenequinonoid pigments), tetrapyrroles (porphyrins, phthalocyanines, chlorins, bacteriochlorins) and fullerenes have been successfully tested [19-21].

Fig. 3. Classes of natural and synthetic photosensitizers used in photodynamic inactivation of microorganisms.

The group of porphyrin derivatives has been prominent, not only because it includes the first formulation approved for photodynamic therapy of cancer [22] but also in the perspective of environmental applications, considering that the use of naturally occurring porphyrins or synthetic related analogues arises as an economical, eco-friendly and human/animal safe option [23].

Porphyrins are a class of heterocyclic aromatic compounds constituted by four subunits of pyrrole type linked by methynic bridges. The presence of these compounds is ubiquitous in nature as part of vital biochemical processes such as oxygen transport (heme group) and photosynthesis (chlorophylls) [24].

If photodynamic therapy is now an established procedure for the treatment of certain non-oncological and oncological diseases [22, 25, 26], it is still not used for infection treatment [15, 27]. In addition, the potential application of PDI to destroy (micro)organisms goes beyond the medical field, with particular focus on the environmental area [28-32].

In PDI studies related with non-clinical applications, artificial white light (halogen or xenon lamps) and sunlight have been used in order to achieve organisms' destruction. The irradiance (or fluence rate) can be given in W m^{-2} , in lux (lx) or in $\mu\text{E m}^{-2} \text{s}^{-1}$ (with E standing for Einsteins in the former terminology, which has been replaced in the new terminology by M, meaning "mole of light"). These light units can be interconverted in the following way: $1 \text{ lx} \approx 9.5 \times 10^{-3} \text{ mW cm}^{-2} \approx 1.8 \times 10^{-3} \mu\text{M m}^{-2} \text{s}^{-1}$ [33]. For example, 100 - 150 lx may represent a shady room in natural light, 30,000 - 40,000 lx an overcast summer's day and 100,000 lx a very bright summer's day [34]. Since the experimental conditions described in literature reports are quite different, sometimes the results cannot be directly compared. The same light dose (J cm^{-2}) can be achieved by varying the light irradiance, the irradiation time or both [21]. However, the effectiveness of the results may be different when using a high irradiance over a short time period or a low irradiance over a longer time, even though the light dose is the same in each case [21, 35]. In general, the PDI of microorganisms is more extensive when higher irradiance and longer treatment duration are used [36]. Increasing the duration of irradiation will improve the yield of treatment, compensating a low concentration of PS or a less efficient PS type [37].

Due to its multi-target nature, and therefore low probability of triggering the development of resistance in (micro)organisms [38-41], this therapy has been tested in various research areas as an alternative approach to actual methods to control insect pests, water quality, microbiological food quality; and also in the disinfection and sterilization of materials and surfaces in different contexts (industrial, household and hospital). Furthermore, the use of

this form of eradicating microbes or noxious organisms becomes increasingly achievable in practice if one thinks that, for certain purposes, the PS may be immobilized on inert solid supports allowing their reuse and recycling, making this technology of broad-spectrum activity, economic, sustainable, durable, and environmentally friendly. To this extent, with the great development of nanotechnology and materials chemistry, several different supports have been created to immobilize a series of PS designed for photoinduced oxygenation reactions [42].

The aim of this review is to present the efforts made in the last decade in the investigation of PDI of (micro)organisms beyond the medical field, using porphyrins as PS, either free or immobilized in solid supports. All the organisms which have been used on PDI experiments are listed in Table 1. The porphyrinic PS reported in these studies are listed in Table 2.

Table 1. List of organisms used in non-clinical photodynamic inactivation experiments

Organism	Organism type	Reference
<i>Acinetobacter baumannii</i>	Bacterium (Gn)	[43]
<i>Acremonium spp.</i>	Fungus (mold)	[44]
<i>Acremonium strictum</i>	Fungus (mold)	[45]
<i>Aedes aegypti</i>	Mosquito (dengue vector)	[46, 47]
<i>Aedes caspius</i>	Mosquito	[48]
<i>Aeromonas salmonicida</i>	Bacterium (Gn)	[49]
<i>Alternaria alternata</i>	Fungus (mold)	[45]
<i>Alternaria spp.</i>	Fungus (mold)	[44]
<i>Anopheles arabiensis</i>	Mosquito (malaria vector)	[50]
<i>Anopheles gambiae</i>	Mosquito (malaria vector)	[50]
<i>Anopheles sp.</i>	Mosquito (malaria vector)	[51]
<i>Artemia franciscana</i>	Crustacea (Branchiopoda)	[52]
<i>Ascaris lumbricoides</i>	Helminth	[36]
<i>Aspergillus flavus</i>	Fungus (mold)	[7]
<i>Aspergillus spp.</i>	Fungus (mold)	[44]
<i>Bacillus cereus</i>	Bacterium (Gp)	[53-58]
<i>Bactrocera oleae</i>	Fly (olive fly)	[59]
<i>Baculovirus</i>	Enveloped DNA virus	[60]
<i>Candida albicans</i>	Fungus (yeast)	[61-63]
<i>Ceratitis capitata</i>	Fly (Mediterranean fruit fly)	[59, 64]
<i>Chaoborus crystallinus</i>	Insect larvae (Diptera)	[23, 65]
<i>Chaoborus sp.</i>	Insect larvae (Diptera)	[66]
<i>Colpoda inflata</i>	Protozoan (Ciliophora)	[52]
<i>Culex pipiens</i>	Mosquito	[67, 68]
<i>Culex quinquefasciatus</i>	Mosquito	[46]
<i>Culex sp.</i>	Mosquito	[66]

Cultivable bacteria from aquaculture water	Bacteria	[49]
<i>Daphnia</i> sp., <i>Daphnia magna</i>	Crustacea (Branchiopoda)	[52, 66]
<i>Edwardsiella ictaluri</i>	Bacterium (Gn)	[69]
<i>Enterococcus faecalis</i>	Bacterium (Gp)	[18, 49, 70]
<i>Escherichia coli</i>	Bacterium (Gn)	[18, 41, 49, 63, 70-92]
Faecal coliforms	Bacterium (Gn)	[37, 93]
Faecal enterococci	Bacterium (Gp)	[37, 93]
<i>Flavobacterium columnare</i>	Bacterium (Gn)	[69]
<i>Fusarium avenaceum</i>	Fungus (mold)	[7, 45]
<i>Fusarium culmorum</i>	Fungus (mold)	[94]
<i>Fusarium poae</i>	Fungus (mold)	[94]
<i>Fusarium</i> spp.	Fungus (mold)	[44]
<i>Ichthyophthirius multifiliis</i>	Protozoan	[65, 95]
Influenza virus strain X-31, A/Aichi/2/68 (H3N2)	Virus	[96]
<i>Legionella pneumophila</i>	Bacterium (Gn)	[97, 98]
<i>Liriomyza bryoniae</i>	Fly (leafminer fly)	[99]
<i>Listeria monocytogenes</i>	Bacterium (Gp)	[53, 57, 80, 100-103]
<i>Mucor</i> spp.	Fungus (mold)	[44]
<i>Musca domestica</i>	Fly (house fly)	[68]
<i>Mycelia sterilia</i>	Fungus (mold)	[44]
<i>Mycobacterium smegmatis</i>	Bacterium (Acid-fast)	[75]
<i>Parasarcophaga argyrostoma</i>	Fly (flesh fly)	[104]
<i>Penicillium chrysogenum</i>	Fungus (mold)	[105]
<i>Penicillium</i> spp.	Fungus (mold)	[44]
<i>Photobacterium damsela</i> subsp. <i>damsela</i>	Bacterium (Gn)	[49]
<i>Photobacterium damsela</i> subsp. <i>piscicida</i>	Bacterium (Gn)	[49]
<i>Planktothrix perornata</i>	Cyanobacterium	[69]
<i>Polyomavirus</i>	Virus (Non-enveloped DNA virus)	[60]
<i>Pseudomonas aeruginosa</i>	Bacterium (Gn)	[43, 83]
<i>Pseudomonas</i> sp.	Bacterium (Gn)	[49]
<i>Rhizopus oryzae</i>	Fungus (mold)	[7, 45]
<i>Rhizopus</i> spp.	Fungus (mold)	[44]
<i>Saccharomyces cerevisiae</i>	Fungus (yeast)	[8]
<i>Salmonella enterica</i>	Bacterium (Gn)	[102, 106]
<i>Salmonella</i> sp.	Bacterium (Gn)	[80]
<i>Saprolegnia</i> spp.	Fungus (mold)	[81]
<i>Selenastrum capricornutum</i>	Green alga	[69]
<i>Staphylococcus aureus</i>	Bacterium (Gp)	[43, 49, 69, 73-75, 77-83, 86, 87, 91, 107, 108]
<i>Stomoxys calcitrans</i>	Fly (house fly)	[109]
T4-like phage	Bacteriophage	[17, 38, 70]
<i>Taenia</i> sp.	Helminth	[36]
<i>Tetrahymena thermophila</i>	Protozoan (Ciliophora)	[52]
<i>Trichothecium roseum</i>	Fungus (mold)	[7]
<i>Ulocladium oudemansii</i>	Fungus (mold)	[8]

<i>Vibrio anguillarum</i>	Bacterium (Gn)	[49]
<i>Vibrio fischeri</i>	Bacterium (Gn)	[110, 111]
<i>Vibrio parahaemolyticus</i>	Bacterium (Gn)	[49]

Gn, Gram-negative; Gp, Gram-positive

2. Applications on the environment, water and foodstuff

2.1 Insect pest elimination

Alternative pesticides for pest and vector control have been required for more than a decade. Insecticide industry and vector control management face several problems nowadays: development of resistance in major vectors to common insecticides; abandonment of certain compounds for safety reasons and environmental and human health concerns about the use of many older generation insecticides, such as DDT; economic factors and market constraints; lack of available, safe and cost-effective larvicides for addition to drinking water; depletion of safe and cost-effective insecticides; re-emergence of disease caused by the early efforts to reduce indoor residual insecticide application rates and increase of vector-borne disease transmission [112-114].

Therefore, there is an urgent need for improved, novel, cost-effective insecticides and insecticide treated materials; long-lasting insecticide formulations for such materials and sprays; economic feasibility and commercialization analyses for pesticides development and, most importantly, providing solutions to overcome the increasing problems of resistance to current insecticides. Accordingly, it has been suggested the development of insecticide combinations, such as bi-treated nets and insecticide treated materials [112], or new active ingredients based on novel modes of action [115].

The idea of using PS as photopesticides (or photoinsecticides) is not new [116, 117]. However, the use of porphyrins for this purpose was only mentioned from the late 80s of the last century [118-121]. Since then, the use of natural or synthetic porphyrin derivatives has been increasingly exploited to control and eradicate various types of insects, including pest flies capable of inducing significant damage to agricultural crops, and mosquitoes vectors of pathogens responsible for malaria (*Anopheles*), yellow fever (*Culex*, *Aedes*) dengue fever (*Aedes*) and encephalitis (*Aedes*, *Culex*, *Anopheles*) [46-48, 50, 66-68]. Nowadays, there is a global distribution of these organisms. In the case of *Aedes aegypti*, for example, its sporadic presence has been recognized in mid-latitudes such as in Britain, France, Italy, Spain, Malta, Portugal and other European countries [122]. Recently, in Madeira island (Autonomous Region of Madeira), Portugal, there was a dengue outbreak starting in October 2012 with 2170 cases

of dengue fever notified until early April 2013. In mainland Portugal, 11 cases have been reported and 71 cases in thirteen European countries, all in travelers returning from the island, and none of them lethal [123]. Current updates of first detection or confirmation of the presence of other disease vectors can be found at the European Mosquito Bulletin website [124].

In the photosensitized insect eradication, PS which are already registered as food additives, such as chlorophylls, chlorophyllins and their copper complexes [125, 126], or phototherapeutic agents as hematoporphyrin IX (HpIX) or 5-aminolevulinic acid (5-ALA), used as a precursor of protoporphyrin IX (PPIX) [22], have been especially successful. They have a low cost production, lack of mutagenic activity, high efficiency and high level of safety to humans and, in general, to other mammalian species [22, 127].

Typically, experimental designs of laboratory studies, or studies under semi-field conditions, use fly larvae or adult mosquitoes, laboratory-reared or collected in breeding sites, which are brought into contact with solutions containing free-base porphyrins or porphyrin incorporated into baits. An initial period of dark incubation, more or less extended to allow the uptake/ingestion of PS is followed by irradiation with natural or artificial sunlight and determination of the mortality percentage or the median lethal dose (LD_{50} , i.e., the dose at which death is produced in 50% of the experimental organisms) (Fig. 4). Many studies also inspect the amount of porphyrin taken up, the anatomical site where it binds and its clearance.

Hematoporphyrin IX (HpIX) has been tested on *Ceratitis capitata* (Mediterranean fruit fly) and *Bactrocera oleae* (olive fly) [59]. A sugar/protein bait containing 8 μ M HpIX led to 100% mortality of *C. capitata* and *B. oleae* flies within the first day after 1 h and 2 h of exposure to 2080 μ E $s^{-1} m^{-2}$, respectively. However, a milder irradiance such as 760 μ E $s^{-1} m^{-2}$ was also found effective in decreasing the number of surviving flies when the irradiation time was prolonged to 2 h. The lower photosensitivity of *B. oleae* flies was possibly due to the smaller amount of ingested HpIX and/or to its darker pigmentation. HpIX was localized in the cuticle, the midgut, the Malpighian tubes and the adipose tissue of the flies [59]. The same PS was also tested on *Stomoxys calcitrans* (house fly) [109]. Flies fed with a concentration range from 4.7 to 7.5 μ M of HpIX and irradiated for 1 h at an irradiance of 1220 μ E $s^{-1} m^{-2}$ underwent total mortality after 2 – 3 days. Only at the highest HpIX dose, a significant percentage of dead flies was observed, for the 1 h of light exposure. This insect has a more darkly pigmented body and larger size than *C. capitata* and *B. oleae*. Besides HpIX, other *meso*-substituted porphyrins, with two, three and four positive or negative charges have been used for insect photosensitization [109, 128]. The most efficient porphyrin was 5,10-bis(1-methylpyridinium-

4-yl)-15,20-diphenylporphyrin (Di-Py⁺-Me-Di-Ph_{adj}), a dicationic amphiphilic porphyrin which at the concentration of 0.85 mM caused 100% mortality on *C. capitata* within 1 h of irradiation at an irradiance of 1220 $\mu\text{E s}^{-1} \text{m}^{-2}$. The photoinsecticidal efficiency of porphyrins seems to increase with the increasing hydrophobicity of the molecule, either by the reduction in the overall number of positive or negative charges or by the replacement of the 1-methylpyridinium moiety with phenyl rings.

Fig. 4 Schematic illustration of the *in vivo* PDI experiments on disease-transmitting insect vectors, contaminated foodstuff and infected fish in aquaculture. PS: photosensitizer.

The factors that appear to affect the efficiency of insects' photosensitivity by porphyrins have been identified by Ben Amor et al. [59, 109, 128], serving as a starting point for designing new strategies for treatment optimization and specificity increase. Such factors are light irradiance, total light dose, PS chemical structure (higher photosensitivity associated with higher degree of hydrophobicity of the porphyrin, particularly obvious with the amphiphilic ones), concentration of porphyrin in the bait, thickness and color of the insect integument, and clearance from the organism in a 24 - 48 h time interval after the porphyrin uptake [129].

HpIX and three more porphyrin derivatives were used in the PDI of different mosquito larvae under field conditions. A HpIX LD₅₀ of 3.2 mg L⁻¹ was established for the fourth instar of *C. quinquefasciatus* and this porphyrin was the only effective one on *A. aegypti* larvae [46].

The strong and fast photo-larvicide activity of HpIX against *C. capitata* immature larval stages has also been reported [64]. The LD₅₀ of HpIX in the food, when activated by light (47 photons $\mu\text{mol s}^{-1} \text{m}^{-2}$), was 0.173 mM, determined in the period entailed from egg hatching to adult ecdysis. The corresponding HpIX LD₅₀ during the dispersal period alone was 0.536 mM. HpIX elicited a mortality of 90.87%, which was mainly concentrated during prepupal and early pupal stages. Loci in the brain and in the gut were damaged by ROS [64].

Awad et al. [67] demonstrated the efficiency of HpIX and of a formulation based on HpIX powder, sugar and other additives, as larvicidal substances on *Culex pipiens* Egyptian field strains, exposed to 3, 9 and 18 h of natural sunrise. A HpIX concentration of 10 μM and 100 μM decreased the larval survival by 94% and 99.3% at the end of 5 days, respectively. On the other hand, concentrations of 1 μM and 10 μM of HpIX-formulation caused a decrease in larval survival of 92.7% and 99.2%, respectively, after 5 days. It was proposed that a synergistic effect occurred due to the incorporation of sugar and other additives to the HpIX, which could reflect the suitability of using the sugar as HpIX carrier in the commercial formula [67].

El-Tayeb et al. [104] studied the effect of HpIX on the flesh fly *Parasarcophaga argyrostoma* in adult stage. A concentration of 10 mM of HpIX caused a mortality of 83% and 96% of the treated flies after exposure to natural sunlight with an irradiance of 236.5 and 1935 W m⁻², respectively. Histological studies showed the high ability of HpIX to accumulate in the insect organs and to cause high extent damage in the alimentary canal tissue. The increase in irradiance of light and in irradiation time enhanced fly mortality. So, although the most efficient HpIX concentration to control *P. argyrostoma* was 10 mM, concentrations of 1mM and 0.01 mM were sufficient to control *Musca domestica* and *Culex pipiens*, respectively [68]. More recently, larvae of the mosquito *Aedes caspius* have been efficiently photoinactivated using 1 mM of HpIX-formulation. The larval mortality has improved by increasing light irradiance and exposure times [48].

In order to be applied to endemic areas with scarce economic resources, PS must be inexpensive. Some researchers have used natural and modified PS centered on chlorophyll derivatives (chlorophyllin and pheophorbide) and sunlight. *Chaoborus* sp., *Daphnia* sp. and *Culex* sp. larvae were photosensitized with chlorophyllin (15 mg L⁻¹), incubated in darkness overnight and were then irradiated for 3 – 4 h with artificial sunlight (PAR: 149.66 W m⁻², UV-A 32.67 W m⁻² and UV-B 0.77 W m⁻²). After incubation, chlorophyllin eliminated the different

organisms at remarkably low concentrations. The LD₅₀ value in *Culex* sp. larvae was about 6.88 mg L⁻¹, in *Chaoborus* sp. larvae of about 24.18 mg L⁻¹, and in *Daphnia* 0.55 mg L⁻¹. It was found that during the puparium, mosquito larvae are relatively insensitive to chlorophyllin treatment and, during metamorphosis, the chrysalid is encapsulated and totally stops food uptake. Probably because of that, the accumulation of chlorophyllin inside the organism is limited and the photodynamic effect is reduced. As well as in other studies, some dark toxicity has also been observed. Other small animals, like *Daphnia* and fish (*Chaoborus*) larvae are also affected by chlorophyllin [66]. On the other hand, unlike fish larvae, more mature fish are unharmed and survive chlorophyllin treatment at concentrations, where, for example, *Culex* larvae are severely affected. This has been shown in field tests in Nigeria in which chlorophyllin was used on *Anopheles* larvae and successfully destroyed the mosquito larvae in a treated pond, without harming any of the other aquatic organisms [51]. Moreover, in addition to the inactivation of mosquito larvae, Wohllebe et al. [65] demonstrated for the first time that the photodynamic treatment of *C. crystallinus* larvae with 24 mg L⁻¹ of chlorophyllin solution gives a LC₅₀ with 0.26 MJ (PAR + UV-A + UV-B) and induces necrosis and apoptosis in these organisms. Chlorophyllin was orally taken up and accumulated in the intestine (a dose of 3.2 mg L⁻¹ chlorophyllin with 3 h irradiation induced apoptosis in the intestinal cells) [65].

Besides chlorophyllin, magnesium chlorophyllin, zinc chlorophyll and copper chlorophyll have been tested on *C. crystallinus* larvae [23]. After 6 h of dark incubation and 3 h of light exposure (360 W m⁻²), the LD₅₀ value of magnesium chlorophyllin was about 22.25 mg L⁻¹ and for zinc chlorophyll 17.53 mg L⁻¹. Copper chlorophyll (LD₅₀ 0.1 mg L⁻¹) was shown to be toxic also without light. Chlorophyllin (LD₅₀ 14.88 mg L⁻¹) was lyophilized immediately after extraction, and its photodynamic efficiency remained constant over a 30-day period, representing an increase in photodynamic efficiency of 50% compared to magnesium chlorophyllin isolated with standard methods (no lyophilization) and 18% compared to zinc chlorophyllin. The results showed that about 30 W m⁻² of solar radiation, which is less than 10% of full sunlight is, was sufficient to induce lethal photodynamic effects in larvae (around 30% of the mosquito larvae). Depending on the attenuation in a water body, photodynamic action can also take place below the water surface. Temperature has also been shown to influence the active chlorophyllin uptake by the larvae [23].

As well as HpIX and chlorophyllin, the hematoporphyrin derivative dimethyl ether (HpDE) has also been shown to be a high potential photopesticide against the larvae of the leafminer fly *Liriomyza bryoniae* [99]. The insects exposed to a sugar bait containing 25 mM of HpDE and irradiated for 30 min with broad spectrum visible light at an irradiance of 30 mW cm⁻²

² (54 J cm⁻² light dose), died (100% mortality) 1 day after irradiation (in the case of the females) and 4 days after the irradiation (in the case of the males). The observed differences in the mortality kinetics between female and male flies were possibly due to the body size and biological activity of females [99]. The treatment efficiency strong also strongly depended on the type of PS used, explaining the different attractiveness of the insects for the bait according to the specificity of the PS contained on it [99]. In line with this need, different baits containing porphyrin to inactivate disease-transmitting vectors have recently been designed and tested.

Lucantoni et al. [47] prepared a formulation constituted by 5-(1-tetradecylpyridinium-4-yl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin (Tri-Py⁺-C₁₄-Py⁺-Me) and powdered food pellet (PFP). First, the LC₅₀ of C₁₄ porphyrin in solution was tested on photosensitized 3rd – early 4th instar *A. aegypti* larvae. LC₅₀ values of 0.1 μM (0.15 mg L⁻¹) and 0.5 μM (0.77 mg L⁻¹) were obtained after irradiation intervals of 12 h and 1 h, with 4.0 mW cm⁻² artificial white light, respectively. Tri-Py⁺-C₁₄-Py⁺-Me was shown to be active after ingestion by the larvae and caused irreversible lethal damage to their intestinal tissues (midgut and caecal epithelia). The porphyrin carrier formulation (25 mg of PFP in 500 mL of 50 μM of Tri-Py⁺-C₁₄-Py⁺-Me – PF-50-Tri-Py⁺-C₁₄-Py⁺-Me) and a 5 μM of Tri-Py⁺-C₁₄-Py⁺-Me solution were both 100% effective up to two weeks, and the amount of Tri-Py⁺-C₁₄-Py⁺-Me required to prepare the PF-50-Tri-Py⁺-C₁₄-Py⁺-Me was 10 times smaller than the Tri-Py⁺-C₁₄-Py⁺-Me required to treat the incubation medium. In the same direction, Fabris et al. [50] associated 5-(1-dodecylpyridinium-4-yl)-10,15,20-tris(1-methylpyridinium-4-yl)porphyrin (Tri-Py⁺-C₁₂-Py⁺-Me) with two distinct carriers: a model of a pharmaceutical oral vehicle (Eudragit® S100, EU) and a model of foodstuff carrier (cat food pellet Friskies®, CF). These porphyrin-carrier formulates (50 μM Tri-Py⁺-C₁₂-Py⁺-Me dose) were tested against overnight fed *Anopheles gambiae* and *Anopheles arabiensis* larvae, exposed to sunlight (30 - 110 mW cm⁻²) for 0.5 - 3 h. These conditions led to high photoinactivation efficiency against laboratory reared *A. gambiae* and *A. arabiensis* larvae (almost complete mortality). On wild (field-collected) larvae, the formulation EU-50-Tri-Py⁺-C₁₂-Py⁺-Me EU caused 100% mortality on *A. gambiae* M and S forms but CF-50-Tri-Py⁺-C₁₂-Py⁺-Me showed variable results depending on the site where the larvae were collected. Not only the association of the PS with suitable carriers promoted a fast and selective internalization of formulates by the *Anopheles* larvae which guaranteed their death upon subsequent sunlight exposure, but also the nature of the carrier affected the overall efficacy of the porphyrin formulates. This was shown by the administration of a 1:1 mixture of CF-50-Tri-Py⁺-C₁₂-Py⁺-Me and laboratory food for larvae (TetraMin®) inducing an extensive mortality of both laboratory

reared and wild *Anopheles* larvae. These results indicated the primary importance of palatability in the design of oral larvicide formulations [50].

According to Lucantoni et al. [47], an insoluble baited insecticidal formulate should be actively consumed by the larvae; an efficient and cost-effective employment of the PS; suitable for application in household water storages for drinking and other domestic purposes; unlikely to affect the organoleptic properties of the stored water; selected or manipulated on different porphyrin carriers in a way to standardize the particle dimension to a size range that is especially palatable for mosquito larvae (e.g., 5 – 50 μm), thus reducing the risks of uptake by non-target organisms.

Table 2. List of porphyrinic derivatives used in non-clinical photodynamic inactivation experiments

Porphyrin	Immobilized (Reference)
No [47] No [50, 52, 69]
<div> <div> Tri-Py⁺-C₁₂-Py⁺-Me </div> <div> ξ-(CH₂)₁₁CH₃ </div> </div> <div> <div> Tri-Py⁺-C₁₄-Py⁺-Me </div> <div> ξ-(CH₂)₁₃CH₃ </div> </div>	<div> <div>R</div> <div>ξ-(CH₂)₁₁CH₃</div> </div> <div> <div>ξ-(CH₂)₁₃CH₃</div> </div>

			R¹	R²
Mono-Py⁺-Me-Di-mes_{opp}-NH₂
Tri-Py⁺-Me-NH₂
Tri-SO₃⁻-NH₂
TPPNH₂

On filter paper [82]
On cotton fabric
[86, 108]

	M	R¹	R²
ZnTri-Py⁺-Me-CCH	Zn(II)
Tri-Tolyl-OCCH	2H
Tri-Tolyl-CO₂H	2H
Tri-Tolyl-Py	2H
Tri-Tolyl-OC₁₀CO₂H	2H

On polysilsesquioxane plastic films [61]
On cotton fabric [87]
On azide-modified cellulose nanocrystals [43, 75]
On chloroacetyl cellulose ester chlorides [78]
On cellulose laurate esters plastic films [79]

No [71] No [93] No [49, 71, 93, 110] and Tri-Py ⁺ -Me-PF on silica coated Fe ₃ O ₄ nanoparticles [70] and also on CoFe ₂ O ₄ nanoparticles [111]
Tri-Py⁺-Me-PF			No [81] No [105] No [36, 37, 71, 74, 81, 105]; Tetra-Py ⁺ -Me was entrapped into microporous silica gels [88] and into three alkylene-bridged polysilsesquioxanes [89] On optically transparent indium tin oxide electrodes [63]	
	Tri-Py⁺-Me-CO₂Me

Tetra-Py⁺-Me			On chitosan membranes [72] Pd(II) TetraTPPCO ₂ H On a polyurethane matrix [83]	
	Tetra-Py⁺-C₅
	Tetra-Py⁺-C₁₂
	Tetra-Py⁺-C₁₄

Tetra-<i>N,N</i>-DiPh	M 2H
	Pd(II)Tetra-<i>N,N</i>-DiPh	Pd(II)			
	TetraTPPOH		R	M 2H	
	TetraTPPNH₂		R	M 2H	
	Pd(II)TetraTPPCO₂H		R	M Pd(II)

<p>Tetra-F₄-SPy</p> <p>Tetra-F₄-SPy⁺-Me</p>	<p>R</p>	<p>No [105]</p>
<p>TPP M= 2H SbTPP M= Sb(OH)₂</p>		<p>In hydrophilic polycaprolactone and polyurethane (TecophilicH) nanofibers [60]; on electrospun polymeric nanofiber materials polyurethane Larithane™, polystyrene, polycaprolactone, and polyamide 6 [76]; into three alkylene-bridged polysilsesquioxanes [89]; and in a silica-gel supported antimony porphyrin complex SbTPP [97, 98]</p>
<p>5-ALA</p>		<p>No [44, 53, 99, 100, 102]</p>

<table><tr><th></th><th>R</th></tr><tr><td>HpIX</td><td>$\sim$-OH</td></tr><tr><td>HpDE</td><td>$\sim$-OCH₃</td></tr></table>		R	HpIX	$\sim$ -OH	HpDE	$\sim$ -OCH ₃	No [7, 8, 45, 99, 130]						
	R													
HpIX	$\sim$ -OH													
HpDE	$\sim$ -OCH ₃
<table><tr><th></th><th>M</th></tr><tr><td>PPIX</td><td>2H</td></tr><tr><td>Zn(II)-PPIX</td><td>Zn(II)</td></tr></table>		M	PPIX	2H	Zn(II)-PPIX	Zn(II)	No [94]; on acid-functionalized multi-walled carbon nanotubes [96, 107]; on cellulose laurate esters plastic films [77]; and on nylon fibers [73]						
	M													
PPIX	2H													
Zn(II)-PPIX	Zn(II)
<table><tr><th></th><th>M</th><th>R</th></tr><tr><td>NaChl</td><td>Mg(II)</td><td>$\sim$-CH₃</td></tr><tr><td>E-140</td><td>Mg(II)</td><td>$\sim$-CH₃ / $\sim$-CHO</td></tr><tr><td>E-141</td><td>Cu(II)</td><td>$\sim$-CH₃ / $\sim$-CHO</td></tr></table>		M	R	NaChl	Mg(II)	$\sim$ -CH ₃	E-140	Mg(II)	$\sim$ -CH ₃ / $\sim$ -CHO	E-141	Cu(II)	$\sim$ -CH ₃ / $\sim$ -CHO	No [56-58, 101-103] and on chitosan [106] (E-140 and E-141) On gelatin films and coatings [80]
	M	R												
NaChl	Mg(II)	$\sim$ -CH ₃												
E-140	Mg(II)	$\sim$ -CH ₃ / $\sim$ -CHO												
E-141	Cu(II)	$\sim$ -CH ₃ / $\sim$ -CHO
No [92]; on silica-gel and on a Merrifield resin-based material [90]												

2.2 Water disinfection

Water reuse is increasingly becoming an essential requirement. Not only in undeveloped countries but also in developed countries, treating drinking water and wastewater may be

challenging. Chlorine-based agents, the most commonly used water disinfectants, are effective against a broad range of microbes, are inexpensive and easy to use, but lack effectiveness against parasites and lead to toxic by-products. Alternatives to chlorine such as ozone, chloramines, chlorine dioxide, and UV irradiation, also have advantages and drawbacks dealing with cost, efficiency, stability, ease of application, development of microbial resistance and mutations triggered by UV irradiation, and nature of formed by-products [131, 132]. In this way, new technologies for water treatment have emerged in recent years [133].

The PDI of microorganisms in the context of water treatment is considered to cause minimal environmental impact and overcome economic, ecological and public health issues. Contrarily to the conventional methods, no toxic by-products are formed and no microbial resistance is developed. The possibility of immobilizing efficiently PS in insoluble supports is also an advantage of PDI. By this way it is possible to remove the PS after treatment from environmental waters and to reuse it, which turns this technology environmentally friendly and cost-effective. Besides, the use of sunlight as light source in PDI has to be considered in order to establish a sustainable antimicrobial protocol. Since sunlight penetrates deeply into the water column, a nearly uniform illumination can be achieved and high water volumes may be treated. This approach becomes inexpensive since it is based on the use of a low cost visible light source. Moreover, for PS as porphyrins with the Soret absorption band in the 420–430 nm spectral region, there is an efficient interaction with blue light, which has a high penetration depth into natural waters and is the most effective in the visible range in the inactivation of microorganisms by cationic porphyrins [134].

The possibility of treating wastewater for reuse in agriculture (crop irrigation) was mentioned in the research work carried out by Alouini and Jemli. [36, 37] Accordingly, the authors demonstrated the efficient PDI of helminth eggs by the tetracationic 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (Tetra-Py⁺-Me) under visible light illumination, in clear water and in secondary treated wastewater. In addition to different concentrations of PS and light intensities, the influence of turbidity (content of suspended solids), agitation (aeration), concentration of dissolved oxygen and the ultrastructural changes of two types of eggs of parasites was also investigated. Suspensions of *Ascaris lumbricoides* and *Taenia spp.* were irradiated with artificial white light (0 - 0.5 W cm⁻²) with Tetra-Py⁺-Me (5 - 30 μM) for 15 min in clear water and 30 min in wastewater. There was a destruction of around 28% of *Ascaris* eggs with 0.33 W cm⁻² and 10 μM of Tetra-Py⁺-Me, after 15 min in clear water, and after 30 min in wastewater. The increase in the light irradiance to 0.5 W cm⁻² caused 47% of destruction of *Ascaris* eggs in wastewater after 30 min of irradiation. This increase turned

negligible the difference in the sensitivity of *Ascaris* eggs in clear and wastewater. An increase in the oxygenation of the wastewater and in the agitation process improved the photosensitivity of *Taenia* eggs. In fact, these effects were more evident when the concentration of PS was also increased. The process was optimized under the following conditions: dissolved oxygen was maintained at 7 mg L⁻¹, the Tetra-Py⁺-Me concentration at 30 µM and the egg suspension was mixed at 300 rpm. The efficiency of the photoprocess was mainly controlled by the Tetra-Py⁺-Me concentration, the irradiation time and the light intensity. Also, the dissolved oxygen concentration, water quality and the type of eggs can influence the sensitivity of helminth eggs to photosensitization. *Ascaris* eggs were found to be more sensitive to photosensitization than *Taenia* eggs. The same authors also used Tetra-Py⁺-Me in several concentration (1.0 µM, 5.0 µM e 10.0 µM) and sunlight irradiation (1235 mW cm⁻²) up to 240 min to inactivate fecal coliforms and fecal streptococci on secondary wastewater samples. After 60 min, at 5.0 µM and 10.0 µM, a decrease of 2.94 and 2.4 logs in fecal bacteria counts was observed, respectively. After 240 min, a total cell survival reduction (> 4.0 log units) was achieved with both concentrations. The 5.0 µM concentration was considered to be more suitable to reduce fecal coliforms in wastewater since it allows obtaining a good treatment yield and it is more economic [37]. The suspended solids (turbidity) were the most influential solution parameter on the efficiency of the photochemical process. Turbidity reduces light penetration, which reduces the PS excitation and the absorption by the helminth eggs [36]. The decrease in log counts of fecal coliforms was ≈ 1.0 after 1 h of phototreatment by 5 µM Tetra-Py⁺-Me when suspended solids reached 50 mg L⁻¹ [37].

Albeit these results were promising, the practical application of photodynamic treatment to disinfect microbiologically polluted waters depends on many factors: the removal of the PS after photodynamic treatment to avoid the release of PS to the environment; the use of photostable PS (i.e., PS which do not bleach under irradiation); the impact of this procedure on the structure of the natural non-pathogenic microbial communities; the toxicity of the PS to aquatic organisms at doses which induce marked mortality on microbial pathogens; the effect of physical and chemical properties of environmental waters; and the possibility of using sunlight as light source [28, 32].

In a pioneering work, Bonnett et al. [72] incorporated *meso*-tetraarylporphyrins with amino and hydroxy substituents and also a tetra sulfonated zinc phthalocyanine (ZnPcS₄) into chitosan membranes for the specific purpose of water disinfection. Model reactors for a large-scale water-flow system were designed: a static photoreactor system with 7 mL of bacterial suspension (3500 cells mL⁻¹) and a circulating water photoreactor system with 455 mL of

bacterial suspension (10^5 CFU - colony forming units- per mL), representing the significant levels of water contamination. After 30 min of irradiation with white light, the chitosan membrane containing the 5,10,15,20-tetrakis(4-aminophenyl)porphyrin (TetraTPPNH₂) caused a reduction to 1300 CFU mL⁻¹. However, the membrane prepared with ZnPcS₄, was much more effective and was able to completely inactivate the bacteria after 30 min. When the more efficient membrane was stored in the dark for nine months, the photodynamic action was still detectable demonstrating its thermodynamic stability. With that model system, the photoinactivation with immobilized PS can be used to lower microbial levels in water flow systems [72].

The bactericidal effect of silica gel-supported antimony porphyrin complex SbTPP/SiO₂, under visible-light irradiation over *Legionella pneumophila* has been reported by the group of Yasuda et al. [97, 98]. Laboratory and environmental field experiments in a cooling tower of 800 L capacity were carried out using fluorescent lamps as light source, and also in a public fountain filled with 13 m³ of water and sunlight irradiation. *In vitro*, the inactivation of *L. pneumophila* with SbTPP/p-SiO₂ (10 mg) reduced the survival rate to 0.6% after 60 min of irradiation. In the cooling tower, after 10 days, the concentrations of *Legionella* were reduced to the detection limit, and these levels were kept until the irradiation had finished. In the public fountain, the bacterial concentrations were reduced to the detection limit 12 days after the SbTPP/SiO₂ catalyst had been installed in the fountain. The bacterial concentrations were kept at less than 30 CFU 100 mL⁻¹ for 3 months until the removal of the catalyst from the fountain [97, 98].

Pyrrolidine-fused chlorin derivatives (TPFPC) showed, after immobilized on either silica gel or Merrifield resin-based material, significant activity against *E. coli* (3.0 log reductions) with 4.0 mW cm⁻² after 180 min. The photostability of the materials and their preserved activity after 3 successive cycles was envisaged as a promising option for water disinfection [90, 92].

Since 2004, our research group has developed a broad-spectrum of PS, namely cationic porphyrins, which can efficiently inactivate bacteria [18, 71], bacterial endospores [135], viruses [17, 35, 136] and fungi [105]. One of the most effective compounds, a tricationic porphyrin with a pentafluorophenyl group (5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide, Tri-Py⁺-Me-PF), has been tested on bacteria and viruses to check the viability recovery and resistance development after repeated incomplete photoinactivation cycles. The bacteria and viruses that were inactivated to the detection limit with Tri-Py⁺-Me-PF did not recover viability after one week and the resistance is not enhanced

after ten sub-lethal photosensitization treatments [38, 41]. At the initial stage of our work, porphyrins were used to photoinactivate faecal coliforms and faecal enterococci in wastewater samples from a secondary-treated sewage plant [93]. Two of the cationic porphyrins used (Tri-Py⁺-Me-PF and Tetra-Py⁺-Me) inactivated 94 – 99.8% of the faecal coliforms at 5 µM upon white light at low irradiance (4 mW cm⁻²) after 270 min of irradiation, demonstrating high efficiency. In this study, two rapid monitoring methods were used to oversee the bacterial photoinactivation: galactosidase activity as an indicator of the presence of faecal coliforms and leucine incorporation as an indicator of bacterial activity. The advantages of using these methods are their easier and faster performance against the determination of CFU, giving an excellent relation with faecal indicators abundance [93]. From these first studies, the experimental conditions were standardized for testing the antimicrobial photoinactivation *in vitro* of our porphyrins in further studies: cell cultures ranged from 10⁷ - 10⁸ CFU mL⁻¹, porphyrin concentrations ranged from 0.5 to 5.0 µM, white light irradiation had an irradiance of 4 mW cm⁻² and the maximum exposure time was 270 min (64.8 J cm⁻²). The incubation time of the organisms with the porphyrins was 10 min in the dark, previously to irradiation, in accordance with the literature [14]. Specific conditions beyond these will be described on the text.

Later, seven synthetic cationic *meso*-substituted porphyrins with one to four charges were tested on *Enterococcus faecalis* and *Escherichia coli* (10⁷ CFU mL⁻¹). The results showed that the tri- (Tri-Py⁺-Me-PF and Tri-Py⁺-Me-CO₂Me) and the tetra-cationic PS (Tetra-Py⁺-Me) at 5.0 µM were the most efficient ones, reducing *E. coli* survival by 7 log CFU mL⁻¹ after 90 min with Tri-Py⁺-Me-PF and Tri-Py⁺-Me-CO₂Me and after 270 min with Tetra-Py⁺-Me [18]. The complete photoinactivation of bacteria with low light irradiance (4 mW cm⁻²) suggests that PDI of fecal bacteria can be a possibility for wastewater disinfection under natural light conditions.

It was also reported the photoinactivation of a recombinant bioluminescent *E. coli* strain whose light emission decreased more than 4 log with the three porphyrins used (Tetra-Py⁺-Me, Tri-Py⁺-Me-PF and Tri-Py⁺-Me-CO₂Me). These results were achieved both with artificial white light (4.0 mW cm⁻², 64.8 J cm⁻²) and with sunlight (≈ 62 mW cm⁻², 1004.4 J cm⁻²) after 90 to 270 min with 5.0 µM of PS but Tri-Py⁺-Me-PF was the most efficient compound [71]. The same series of cationic porphyrins were also tested on bacteriophages isolated from wastewater, using white light and 5.0 µM of porphyrin [17]. Again, the tetra- and tricationic derivatives inactivated the T4-like phage to the limits of detection (reduction of ≈ 7 log).

As nanotechnology emerges as an opportunity for water treatment purposes [131, 133, 137, 138], new materials based on magnetic nanoparticles with different porphyrins

covalently immobilized have been recently developed, in order to be easily removed from the water matrix, for subsequent reuse [18, 70]. Three different hybrids have been prepared and tested in microcosm conditions, being the multi-charged nanomagnet hybrid based on Tri-Py⁺-Me-PF quite effective against Gram-positive and Gram-negative faecal bacteria (5 log decrease for *E. faecalis* and *E. coli* at 20 μ M of PS) when using white light irradiation (64.8 J cm⁻²). This hybrid also presented antiviral activity, inactivating the T4-like bacteriophage to the detection limit ($\approx$ 7 log of inactivation) [70].

In order to use this approach in operative field conditions, the multi-charged nanomagnet-Tri-Py⁺-Me-PF hybrid has been recently tested [111]. Preliminary results showed that this hybrid is effective in *Vibrio fischeri* photoinactivation in, at least, a six-cycle reuse, causing a cumulative bacterial reduction higher than 37 log under low light irradiance (4.0 mW cm⁻²).

Also, Tri-Py⁺-C₁₄-Py⁺-Me has been encapsulated within silica microparticles forming a conjugate with *ca.* 0.9 μ m diameter. The conjugate loaded with 12 μ M C14 added to a water sample contaminated with methicillin-resistant *S. aureus* (MRSA) (10⁸ cells mL⁻¹), promoted a tight association of the bacterial cells with the silica microparticle-porphyrin system, and further irradiation with visible light (30 min, 100 mW cm⁻²) caused a 3 log reduction in the survival of MRSA cells in the water sample. The conjugate showed to be stable in aqueous medium for at least 3 months, easily recovered by filtration of the aqueous suspension and kept the high antibacterial photo-activity when reused [91].

Another example of recovery and reuse of immobilized porphyrin used protoporphyrin IX (PPIX) attached to acid-functionalized multi-walled carbon nanotubes (NT-PPIX) as potent antiviral agents to be used on surface disinfection as a coating or in water treatment [96]. Influenza virus strain X-31, A/Aichi/2/68 (H3N2) was irradiated with visible light and 1 mg mL⁻¹ of NT-PPIX up to 90 min. This treatment caused more than a 250-fold reduction in the effective infectious viral dose after a 30 min exposure to light. The percentage of cells that could be infected by 200 ng of virus was only about 1%. A pre-exposure of NT-PPIX for 90 min showed a partial loss of photoactivity, but its effect on the virus was still equivalent to a reduction in the infectious viral dose by almost 50-fold. Reusability studies of NT-PPIX showed to be dependent on photobleaching of the porphyrin moiety and on the yield of recovery of NT-PPIX after each use. Besides being effective bactericidal agents as previously proved on *Staphylococcus aureus* [107], these conjugates may be applied as reusable antivirals in wastewater treatment, as they can be easily recovered without leaving any toxic by-products [96].

Although there is a tremendous need for scientific knowledge on disinfection of hospital wastewater, there is only one report, from our group, on the use of photodynamic treatment on this type of effluent. The efficiency of photoinactivation on four multi-drug resistant isolated strains of *E. coli*, *Pseudomonas aeruginosa*, *S. aureus* and *Acinetobacter baumannii* was evaluated in buffered solution and in hospital wastewater, using 5.0 μM of Tetra-Py⁺-Me and white light (64.8 J cm^{-2}) [139]. The results showed an efficient inactivation of multidrug-resistant (MDR) bacteria in buffered solution (reduction of 6-8 log CFU mL⁻¹) and in the wastewater, in which the photoinactivation of the four bacteria was also effective and the decrease in bacterial number occurred even sooner. This dissimilarity was assigned to dissolved compounds in the hospital wastewater, such as antibiotics.

Aquaculture activity is increasing worldwide, motivated by the progressive reduction of natural fish stocks. This activity suffers, however, substantial financial losses resulting from fish infections by pathogenic microorganisms. The misuse of a wide variety of antibiotics in aquaculture has raised several problems such as the emergence of MDR bacteria in aquaculture environments; the rise in antibiotic resistance in fish pathogens; the transfer of these resistance determinants to bacteria of land animals and to human pathogens; and modifications of the bacterial flora both in water column and in sediments [140]. Moreover, although commercial vaccines against the most common pathogens are available for fish, vaccination is not an option for fish larvae, one of the fish life stages most prone to microbial infection, as it is unfeasible to handle large numbers of these small-sized and frail organisms. Furthermore, fish larvae do not have the ability to develop specific immunity [141]. Consequently, PDI can be an alternative approach to treat fish larvae. The treatment can be applied as a preventive approach against bacterial infections during larvae production, before releasing them in the aquaculture tanks, thereby improving the overall production of adult fish and the sustainability of fish farming.

As a consequence, strategies to control fish pathogens are needed and PDI can be an option to treat diseases and to prevent the development of antibiotic resistance by pathogenic bacteria [142]. As the PS could be degraded and used in a low concentration, it would be innocuous to animals, to fish consumers and to the environment. However, the idea is not to use the PS diluted or dispersed in the aquaculture water, but instead, use it immobilized in solid supports in order that it could be recovered and reused successively, without being discarded. Aquaculture water treated with immobilized PS under sunlight makes this approach attractive, cost effective and eco-friendly.

The photodynamic disinfection of water from fish farms as well as the prevention and treatment of localized fungal infections (saprolegniosis) in fish, with porphyrins as photosensitizing agents, was presented in 2006, demonstrating the inactivation *in vitro* of pathogenic fungi and bacteria, and the photo-treatment of spontaneously or artificially infected fish in a pilot aquaculture pond [81]. The PS chosen were Tetra-Py⁺-Me and Tri-Py⁺-C₁₄-Py⁺-Me (0.05 to 10 μM). The treatment of infected rainbow trout (*Oncorhynchus mykiss*) consisted in two protocols: preventive and curative (Fig. 4). In the preventive protocol, artificially infected fish in 1000 L capacity tanks were dark incubated for 10 min with 0.2 μM of Tri-Py⁺-C₁₄-Py⁺-Me and were not irradiated, or were incubated for 10 min with 0.44 μM of Tetra-Py⁺-Me and irradiated for 1 h with white light (50 mW cm⁻²). The incubation and irradiation treatment was repeated daily, for ten consecutive days, starting from the first day after the infection. In the curative protocol, infected fish were dark incubated with 0.44 μM Tetra-Py⁺-Me for 10 min in an 80 L pool, irradiated for 1 h and moved to a 1000 L tank (treatment repeated daily for six consecutive days); or dark incubated with 0.4 μM of Tri-Py⁺-C₁₄-Py⁺-Me for 24 h in a 150 L tank, not irradiated and then moved to a 1000 L tank [81].

Experiments with *Saprolegnia* ($\approx 10^8$ zoospores mL⁻¹), indicated that Tri-Py⁺-C₁₄-Py⁺-Me caused a decrease of the survival of the fungal cells by about 6 logs, after phototreatment with 10 μM [81]. The preventive protocol determined a reduction of the infected percentage to 10% and 13%, respectively, after one week, with irradiated Tetra-Py⁺-Me and unirradiated Tri-Py⁺-C₁₄-Py⁺-Me, respectively. After two weeks, all the infected individuals from all the experimental groups recovered from the infection. With the curative protocol, a complete remission of the infection was induced within one week, followed by the complete healing of the ulcerated lesion. Gradual photobleaching of both porphyrins was observed but photodegradation products were not toxic [81].

In the case of treating infected fish by this approach, either in a preventive or curative way, the PS was added to the water tank followed by irradiation with artificial white light [81]. In that case, the porphyrins were able to interact with the pathogen cells that had colonized the lesion in fish and either cause their inactivation or prevent their proliferation. The induced-infections by *Saprolegnia sp.* in trouts were external, on the dorsal region, thus the infection was treated by exposing the lesion in the fish to water added with porphyrin and after a period of irradiation.

For aquaculture water disinfection using PDI, the PS may be added to water, in a solid membrane or dispersed in particles to disinfect this matrix before being in contact with fish. Microbiologically contaminated water would be decontaminated prior to being delivered to

the fish tank culture. However, treating fish diseases through PDI is another concern that has not been object of enough scientific research.

Studies carried out in our laboratory indicated that nine bacterial species (*Vibrio anguillarum*, *V. parahaemolyticus*, *Photobacterium damsela* subsp. *damsela*, *P. damsela* subsp. *piscicida*, *Aeromonas salmonicida*, *E. coli*, *Pseudomonas* sp., *S. aureus* and *E. faecalis*) isolated from fish-farming plant water are effectively inactivated (up to 7 log) with the tricationic porphyrin Tri-Py⁺-Me-PF at 5.0 µM, under a low light irradiance (4.0 mW cm⁻²) [49]. The cultivable fraction of the heterotrophic bacteria of the same aquaculture plant, including pathogenic and non-pathogenic bacteria, was also inactivated by PDI, but the efficiency of the inactivation varied during the sampling period. The seasonal variation of photoinactivation efficiency can be due to differences in bacterial community structure but also due to variations of the water physico-chemical properties. The results clearly show that it is more difficult to photoinactivate the complex natural bacterial communities of aquaculture waters than pure cultures of bacteria isolated from these waters [49]. As PDI is not selective for pathogenic microorganisms, the non-pathogenic microbial community of aquaculture waters can also be affected. As non-pathogenic bacteria have an important ecological role in the biogeochemical cycles in aquaculture waters, namely in semi-intensive systems, a careful evaluation of the environmental impact must be conducted before PDI implementation in these systems. Preliminary results from our group showed that not all bacterial populations are affected by PDI. In aquaculture water treated with Tri-Py⁺-Me-PF and exposed to light, a reduction on the number of bacterial genotypes relatively to the non-treated water samples has been observed, indicating that dominant bacterial populations were affected by PDI. However, bacterial population is also affected by simple light exposure [49].

The effect of physico-chemical properties of aquaculture waters on PDI efficiency has also been evaluated [110]. The PDI assays were designed having into account the annual variability of pH (6.5 - 7.5), temperature (13 - 22 °C), salinity (10 - 35 g L⁻¹) and oxygen concentration (2 - 6 mg L⁻¹) values in the fish farms where the water was collected. To monitor the PDI kinetics, the bioluminescence of *V. fischeri* was measured. The variations in pH, temperature, salinity and O₂ did not significantly affect the PDI of *V. fischeri* (≈ 7 log reduction in all conditions) with 5.0 µM of Tri-Py⁺-Me-PF under white light (4.0 mW cm⁻²). The suspended solids in the aquaculture water reduced the efficiency of PDI in relation to clear aqueous solutions (buffered solution). On the other side, a good response was obtained in aquaculture water by increasing the PS concentration to 20 µM. The kinetics of the photoinactivation of *V. fischeri* in aquaculture water using two different light sources (artificial white light and solar

light) was also tested. The inactivation of *V. fischeri* under solar light (40 mW cm⁻²) was compared to that obtained with artificial white light, using the same total light dose. The results obtained with 20 µM of porphyrin showed that, using the same total light dose (64.8 J cm⁻²), both light sources inactivate *V. fischeri* to the detection limit. As it is intended that this technology will be used in real aquaculture context, using solar irradiation as light source is expected to make this water disinfection approach economically sustainable in terms of energy demand [110].

To overcome the enteric septicemia of catfish, columnaris disease and the presence of odor-producing cyanobacteria in ponds of catfish (*Ictalurus punctatus*) production, the antibacterial and algicidal activity of Tri-Py⁺-C₁₂-Py⁺-Me has been evaluated on the fish pathogens *Edwardsiella ictaluri* and *Flavobacterium columnare* and on *S. aureus* for comparison [69]. The same PS was also used on the odor-producing planktonic cyanobacterium *Planktothrix perornata* and on a representative green alga *Selenastrum capricornutum*. Tri-Py⁺-C₁₂-Py⁺-Me was used in concentrations ranging from 0.01 to 1000 µM and the samples were irradiated with fluorescent lamps at a photon flux density of 16 - 30 µE m⁻² s⁻¹. Minimal inhibitory concentrations were obtained as follows: 9.8 mg L⁻¹ for *E. ictaluri*, 0.5 - 1.0 mg L⁻¹ for *F. columnare* and 0.1 mg L⁻¹ for *S. aureus*. The growth inhibition after 96 h irradiation with 0.07 mg L⁻¹ and 0.2 mg L⁻¹ of C₁₂ porphyrin, inhibited 50% of both *P. perornata* and *S. capricornutum*, respectively (total inhibition was observed at 1.0 mg L⁻¹ in both cases). While *in vivo* tests or other toxicity test were not reported, the preliminary findings showed the broad spectrum activity of Tri-Py⁺-C₁₂-Py⁺-Me and suggested applications include sanitizing empty tanks before restocking and treatment of other aquaculture systems dealing with similar disease outbreaks [69].

Tri-Py⁺-C₁₂-Py⁺-Me, already mentioned as an effective photoactivated antimicrobial agent for aquaculture [69] and disease-transmitting insect vectors [50], has also been tested on the protozoan Ciliophora *Colpoda inflata* and *Tetrahymena thermophile* and on the Crustacea Branchiopoda *Artemia franciscana* and *Daphnia magna*, which are used in routine toxicity assessment in freshwater ecosystems [52]. Following incubation with Tri-Py⁺-C₁₂-Py⁺-Me for 60 min (0.1 – 10 µM range) the organisms were irradiated with visible light (10 mW cm⁻²) for 60 min as well. Tri-Py⁺-C₁₂-Py⁺-Me caused a growth inhibition ≥ 90% against *C. inflata* cysts or trophozoites with 0.3 - 0.6 µM, 3 h after irradiation was ended. In turn, *T. thermophila* vegetative cells required 3 µM for a 50% inhibition of growth, 46 h after irradiation. The complete inactivation of *D. magna* was achieved with 0.6 µM of Tri-Py⁺-C₁₂-Py⁺-Me while in *A. franciscana* photosensitivity was not detected up to 10 µM. Fluorescence microscopy analyses

clearly revealed a damaged morphology induced on cysts of *C. inflata* by 1 μM of PS and on trophozoites of *C. inflata* by 0.6 μM . *T. termophila* also revealed damage with 6 μM of Tri-Py⁺-C₁₂-Py⁺-Me. *D. magna* accumulated the PS in the digestive tract and exoskeleton and *A. franciscana* revealed accumulation especially on the exoskeleton. The resistance of *A. franciscana* to the phototreatment was explained by the very low amount of the ingested PS along with its ability to adapt to extreme conditions. These findings emphasized the need for carefully tailored irradiation protocols, taking into account the nature of the specific water basin, particularly in what refers to its biotic characteristics [52].

Recently, chlorophyllin-mediated PDI has been suggested as a new promising treatment to control ichthyophthiriosis, a white spot-causing disease in many freshwater fish species by the protozoan parasite *Ichthyophthirius multifiliis*. Different life stages of the parasite were tested: trophonts (encysted stage in the host) and tomites (motile, infective stage). Samples were incubated 60 min in the dark with 0.5 - 10 $\mu\text{g mL}^{-1}$, followed by irradiation with simulated sunlight (PAR 149.66 W m^{-2} , UV-A 32.67 W m^{-2} , and UV-B 0.77 W m^{-2}) for 30 min. The photodynamic effect on the cells was evaluated by fluorescence microscope after staining with the fluorescent dye acridine orange. Transmitted light microscopy showed that chlorophyllin completely filled the trophont and destroyed the nucleus and the cell wall. The LC₅₀ value, calculated for trophonts of *I. multifiliis*, was 0.67 $\mu\text{g mL}^{-1}$ with a maximum mortality of about 10% observed in dark conditions. Even at a chlorophyllin concentration of 2 $\mu\text{g mL}^{-1}$ in the medium, 100% of the tomites were dead. The results of the *in vitro* experiments showed that the photodynamically active chlorophyllin reduces the number of parasites already at very low concentrations. For the first time it was demonstrated that the photodynamic treatment of *I. multifiliis* with chlorophyllin is very effective against tomites and tomites of these fish parasite [95].

2.3 Elimination of food-borne pathogens

Conventional thermal methodologies used for food preservation are effective against most of the food-borne pathogens. However, major drawbacks deal with uncontrolled chemical reactions within the food matrix which affects their nutritional value. New technologies for food safety such as ultraviolet irradiation, ionizing radiation or high pressure are only at research stage, most of them fail to inactivate bacterial spores, viruses and biofilms, and also require high investment and specialized equipment [143].

In this sense, PDI emerges as a new tool for food decontamination in a non-thermal way [143]. PDI of microorganisms for foodstuff decontamination, contrarily to the conventional

thermal methodologies, satisfy the increasing consumer demand for microbiologically safe fresh-like foods, with minimal modification of nutritional and organoleptic properties [144]. Moreover, when compared with other non-thermal new technologies for food safety such as high pressure, PDI is effective to inactivate bacterial spores, viruses, microbial biofilms and antimicrobial resistant microorganisms, which are the most common causal agents in bacterial food poisoning outbreaks.

PDI of common food pathogens (yeasts, molds and bacteria) has been reported *in vitro*, and *in vivo* on foodstuff (sprouts, fruits, vegetables, meat products) by directly applying the PS or immobilizing it on edible solid supports (Fig. 4) and also on the surface of packaging material. In the last ten years, representative natural type porphyrins such as HpDEe, PPIX, sodium chlorophyllin (NaChl) or 5-ALA as prophyrin precursor, and also synthetic analogues, have been used in scientific research related with microbiological food control.

HpDE (2.5 - 71 μM) has been tested on several strains of micromycetes with distinct morphological type: *Aspergillus flavus*, *Trichothecium roseum*, *Fusarium avenaceum*, *Rhizopus oryzae* [7], *Alternaria alternata* and *Acremonium strictum* [45], using visible light (30 mW cm^{-2}) and 15 min of irradiation (27 J cm^{-2}), on 10^6 spores mL^{-1} suspensions, incubated for 20 min to 18 h before irradiation. After 18 h incubation with HpDE, dark toxicity inhibited the conidia germination of the different microfungi. Concentrations of 51 μM caused 100% inhibition of spore germination after irradiation of *Aspergillus* [7], *R. oryzae* and *A. alternata* [45]. Dark toxicity percentages of $\approx 15\%$ and 20% were observed at this concentration for both fungi, respectively [45]. The germination of microfungi *F. avenaceum* and *T. roseum* spores were inhibited by 71 μM up to 90 - 100% (with 30% of dark toxicity) [7]. For *A. strictum*, only the highest concentration (108 μM) induced a photoinhibition of 90% with 9% of dark toxicity [45]. This dark toxicity was envisaged as a spore germination inhibitor which could prevent germination until they have been washed or diluted out of the surface [7].

High inhibition of conidia germination was also observed after PDI when using PPIX (1 - 4 μM) on *Fusarium poae* and *Fusarium culmorum* isolated from infected barley seeds. The spores ($\approx 10^5 - 10^6 \text{ mL}^{-1}$) incubated 30 min were irradiated with visible light at 150 W m^{-2} (50 - 200 kJ m^{-2}). At a PS concentration of 1 μM and an illumination dose of 200 kJ m^{-2} , germination decreased to 40% for *F. poae* and 65% for *F. culmorum*. At 4 μM , 96% and 55% of conidia were eliminated for *F. poae* and *F. culmorum*, respectively. Also, at micromolar concentrations, PPIX sensitizes the photo-oxidation of proteins and lipids in hydrated conidia when illuminating the spore suspension with light doses of 50 - 200 kJ m^{-2} , disturbing the permeability of membranes and suppressing spore germination [94].

5-ALA has been stated to improve the nutritive value of food and considered as a possible tool to decontaminate wheat seeds prior to their use into preparation of sprouts, with minimal damage to wheat germination and quality. Wheat (*Triticum aestivum* L. var. Zentos) seeds significantly contaminated with several microfungi were soaked and incubated with 6 mM 5-ALA for 4 h. After this time, the viability of *Mucor spp.*, *Penicillium spp.*, *Aspergillus spp.*, *Rhizopus spp.*, *Acremonium spp.*, *Fusarium spp.*, *Alternaria spp.*, *Mycelia sterilia* and other fungi significantly decreased $\approx 1.0 \log \text{ CFU g}^{-1}$ of seeds. The sensitivity of fungi to 5-ALA treatment varied considerably among fungi. Besides antifungal activity, 5-ALA demonstrated to be a growth stimulator of wheat seedlings and roots, without impairment of the vigor of germination and the viability of seeds. It also caused an increase of the chlorophyll content, favoring the rate of photosynthesis and of the activity of antioxidant enzymes, which can promote cellular detoxification from ROS [44]. The selection of this particular pro-drug took into account the fact that PS can interact with the food matrix. Being colorless and odorless, 5-ALA would not change the organoleptic properties if sprayed on food [53]. Moreover, its water solubility, stable shelf-life, non-bleaching character, simplicity of production and the possibility of being a constituent or food additive, make 5-ALA a potential candidate for the control of food pathogens by photosensitization, without damage to food constituents and unwanted coloration [145].

The efficiency of 5-ALA has been tested *in vitro* against *Salmonella enterica*, *Bacillus cereus* vegetative cells and spores, *Listeria monocytogenes* cells and biofilms and on packaging material (adhered cells/spores and biofilms). The approach of adherence to packaging consisted on soaking or submerging trays of polyolefin (a mixture of polyethylene and polypropylene) in $10^7 - 10^8 \text{ CFU mL}^{-1}$ cells or spores solutions for 30 min, followed by dark incubation up to 60 min, drying 20 min and irradiation with visible light (20 mW cm^{-2}) up to 20 min. *In vitro* results showed that *B. cereus* cells were inactivated by ≈ 6 logs with 3 mM, incubated 60 min and irradiated 20 min, and spores ≈ 3 logs with 7.5 mM after 30 min incubation and 15 min irradiation. Cells adhered to packaging material were inactivated by 4 logs after 10 min incubation with 3 mM porphyrin and 15 min irradiation. Increasing the concentration to 7.5 mM and the incubation time to 30 min caused 2.7 log decrease on spores adhered to packaging [55]. An efficient inactivation (6 log) of *Salmonella enterica* serovar Typhimurium resistant to tetracycline (10^7 CFU mL^{-1}) was achieved after 60 min incubation with 7.5 mM of 5-ALA and 20 min irradiation (24 J cm^{-2}) [54]. In the case of *L. monocytogenes*, *in vitro* tests showed that cells were inactivated by 4 logs with 7.5 mM 5-ALA, incubated 120 min and irradiated 20 min. Cells adhered to packaging material were inactivated by 3.7 logs

after 15 min incubation with 10 mM PS and 15 min irradiation (18 J cm^{-2}). With the same concentration, a 3.1 log decrease in viable cells was observed in biofilms formed in 48 h, incubated 30 min in the dark and irradiated for 15 min. Approximately $5.9 \text{ log CFU cm}^{-2}$ of biofilm-associated cells were adhered onto a plastic coupon. The different effectiveness between photosensitization of biofilms and the cells adhered to the surface of packaging material was explained by the polysaccharide matrix of biofilms acting as a diffusion barrier for PS and reducing its accumulation inside bacteria [53]. The fluorescence intensity analysis of endogenous PS in these three microorganisms revealed that *B. cereus* produces endogenous porphyrins ten times more efficiently than *L. monocytogenes* or *S. enterica* [54, 100], explaining its higher susceptibility to 5-ALA-based photosensitization compared to the other two bacteria.

The photodynamic effect of chlorophyllins applied for inactivating food-borne pathogens was firstly reported by Lopez-Carballo et al. [80] In a way to improve food preservation, the authors developed and applied new antimicrobial PS-containing edible films and coatings based on gelatin incorporating sodium magnesium chlorophyllin (E-140) and sodium copper chlorophyllin (E-141). Cell suspensions of *S. aureus* and *L. monocytogenes* (10^8 CFU mL^{-1}) were spread on the surface of nutritive agar plates. E-140- and E-141-containing gelatin films ($80 \mu\text{g mL}^{-1}$) were placed on the surface of the inoculated nutritive agar plates and irradiated for 5 or 15 min at several light intensities: 10,000, 30,000 and 50,000 lx. Additionally, the inhibitory effect of $80 \mu\text{g mL}^{-1}$ E-140-gelatin films on early-stationary phase cells of *S. aureus* and *L. monocytogenes* inoculated on slices of cooked frankfurters was tested. Frankfurter slices were inoculated with bacterial samples ($50 \mu\text{L}$, 10^6 cells) and after inoculum penetration, the sample surfaces were wrapped in E-140 edible films and exposed to 30,000 lx for 15 min. Chlorophyllin E-140 exerted a greater inhibiting effect than chlorophyllin E-141 on the bacterial viability. Differences in the antibacterial activity of the two chlorophyllins could be due to E-140 having a greater ability than E-141 to generate $^1\text{O}_2$ under the experimental conditions tested. Water soluble E-140 and E-141 reduced *S. aureus* and *L. monocytogenes* viability by 5 log and 4 log, respectively. Both procedures of wrapping or coating the food sample with E-140-containing gelatin films and coatings were successful, although the results of the antimicrobial studies were slightly different. The E-140 gelatin film was placed on the surface of a previously inoculated frankfurter sample, the combined treatment of wrapping with E-140 gelatin film and irradiation produced an approximately 1.5 log fall in the viability of *S. aureus* and *L. monocytogenes*. None of the PS treatments showed effect on the viability of *E. coli* or *Salmonella*. The antimicrobial films with potential application as wraps or castings to

avoid contamination on the surface of fresh and processed products would increase their shelf-life and safety. Additionally, minimal impact on the visual sensory properties of the food has been observed with the low concentrations used of incorporated chlorophyllin [80].

Besides immobilized chlorophyllin derivatives, sodium chlorophyllin (NaChl) has also been tested *in vivo* on the surface of fruits and vegetables [58]. The inactivation of *B. cereus* and aerobic mesophils on nectarines, iceberg lettuce, cherry tomatoes, cauliflowers and cherries, and the impact of the treatment on shelf-life (i.e., disease-free period) of the product were evaluated. *B. cereus* ($\approx 10^7$ CFU mL⁻¹) were inoculated on the surface of these food products and they were soaked in the NaChl solution (0.15 mM) for 5 min, dried for 20 min and irradiated with the visible light (20 mW cm⁻²) for 5 min. A 1.68 - 2.88 log reduction on bacterial survival was observed, depending on the surface structure and the light-surface interaction specificity. A reduction of 1.48 - 2.47 log of naturally distributed aerobic mesophils was also achieved. Also, the shelf-life of all treated products could be extended, in some cases, up to 70% compared to non-treated ones [58]. In another study, strawberries were inoculated with *L. monocytogenes* for 30 min, soaked in 1 mM NaChl for 5 min and irradiated for 20 min with visible light (12 mW cm⁻², 14.4 J cm⁻²). Photosensitization decreased *Listeria* by 98% (1.8 log). Naturally occurring yeasts, microfungi and mesophils were inhibited by 86 (0.86 logs) and 97% (1.7 log), respectively. This photo-treatment extended the strawberry shelf-life by 2 days. The total antioxidant activity of treated strawberries increased 19%. No impact on the amount of phenols, anthocyanins or surface color was detected. The re-growth rate of yeast and fungal survivors slightly decreased. Furthermore, photosensitization did not affect strawberry color or taste [103].

NaChl has also been tested *in vitro* and on surfaces of packaging material, with effective results on *B. cereus*, *Listeria* and *Salmonella enterica*. The experimental approach used in the studies below was the same already described for 5-ALA [53-55]. NaChl (0.075 μ M to 75 μ M) was tested against *B. cereus* (10^7 CFU mL⁻¹) vegetative cells and spores, *in vitro* or attached to the surface of packaging material, incubated 2 - 60 min and irradiated with visible light (20 mW cm⁻²). *B. cereus* vegetative cells and spores were inactivated *in vitro* by 7 log with 0.75 μ M after 2 and 5 min irradiation, respectively, after 2 min incubation in both cases. The 4-log photoinactivation of *B. cereus* cells on the surface of packaging materials required a concentration of NaChl 10-fold higher (7.5 μ M). PDI with 75 μ M of NaChl, 5 min of dark incubation and 5 min irradiation caused 5 log reductions in the concentration of attached endospores. Endospores are more resistant than vegetative cells to photoinactivation, since a higher concentration of Na-Chl is required than for vegetative cells for the same level of

inactivation to be attained [56]. *Bacillus* spores were susceptible to 5-ALA-based PDI as well [55]. The comparison of 5-ALA and Na-Chl based photoinactivation of *B. cereus* indicates some advantages of the later PS; this means that using NaChl can shorten the dark incubation time from 20 to 2 min, reduce the irradiation time from 20 to 5 min and lower the optimal concentration (0.075 μ M versus 7.5 mM for 5-ALA) [145].

Listeria biofilms were totally removed from the surface of packaging material at higher NaChl concentrations and after longer incubation times. *L. monocytogenes* cells were inactivated by 7 log using 0.75 μ M of PS, and 30 min irradiation for a thermo-resistant strain and 5 min for a thermo-sensitive one. Decontamination of packaging material from thermo-resistant *L. monocytogenes* cells adhered to the surface reveals that a NaChl concentration of 0.15 μ M is necessary to reduce the 4 log of bacteria with 15 min irradiation while the same reduction against the thermo-sensitive strain could be achieved within 5 min. With this irradiation time, *Listeria* biofilms were also susceptible to photosensitization and surfaces could be totally cleaned from them when 150 μ M of NaChl is used (4.5 logs) [101]. Additionally, a thermo-resistant strain of *Bacillus* was less susceptible to PDI than the thermo-resistant *Listeria* strain [57].

A comparison between the antimicrobial efficiency of PDI with conventional surface cleaning products indicates that NaChl-based photosensitization is much more effective against *B. cereus* attached to the surface of materials than washing with water or using 200 ppm of sodium hypochlorite [56]. In the case of *Listeria* strains (thermo-resistant or thermo-sensitive), washing with water diminishes pathogens by less than 1 log, 200 ppm of sodium hypochlorite by 1.7 log and NaChl-based photosensitization by 4.5 log [101].

Chitosan, as a natural compound, may replace chemical preservatives and may be used to obtain eco-friendly products but it should not interact with food ingredients or alter food organoleptic features [7]. A chlorophyllin – chitosan complex (with 0.001% NaChl – 0.1% chitosan, NaChl–CHS) has been tested against *Salmonella enterica* ($\approx 10^7$ CFU mL⁻¹) upon visible light irradiation (9.6 mW cm⁻²) for 30 min [106]. The photoinactivation treatment (15 min of incubation with NaChl followed by irradiation) led to a 1.05 log reduction of *S. enterica*. The dark toxicity of the NaChl–CHS complex reduced cell viability by also 1.05 log. An extension of the incubation time to 120 min favored the inactivation of *Salmonella* to 1.39 log. An extremely high antibacterial efficiency was demonstrated after photoactivation with the NaChl–CHS complex (7.3 log reduction of microbial population) after 120 min irradiation. A photoactivated NaChl-CHS complex in a slightly acidic environment was suggested as a useful tool against *S. enterica*. The synergetic antibacterial effect of CHS and NaChl supposedly relies

on the complexation of these two compounds governed by thermodynamic equilibrium and results in a uniform distribution of the short-chain component (NaChl) among the chains of the oppositely charged long-chain component (CHS) present in high excess [106].

More recently, the photodynamic antimicrobial efficiency among 5-ALA, NaChl and a combined treatment of 5-ALA plus NaChl has been tested against *L. monocytogenes* and *S. enterica*. Cells were incubated with NaChl (75 μM – 0.075 μM) or 5-ALA (7.5 mM) and further irradiated with visible light (20 mW cm^{-2}). *Salmonella* was more sensitive to 5-ALA ($\approx$ 6.6 log viability decrease with 7.5 mM, 40 min irradiation) than to NaChl ($\approx$ 2.2 log viability decrease with 75 μM upon 40 min irradiation). *Listeria* was more sensitive to NaChl (7 log drop with 0.75 μM , 5 min irradiation) than to 5-ALA ($\approx$ 3.8 log with 0.75 μM , 20 min irradiation and 60 min incubation). Both bacteria were inactivated to an undetectable level when combined treatment was applied (incubation time of 60 min, with 7.5 mM of 5-ALA and 0.15 μM of NaChl, 40 min of irradiation) [102].

Besides HpDE, PPIX, 5-ALA and NaChl, novel cationic porphyrins were synthesized to be used on food contaminants. As such, the biological evaluation of five cationic porphyrins was tested on *Penicillium chrysogenum* conidia. Two different series of cationic porphyrin groups were prepared from 5,10,15,20-tetrakis(4-pyridyl)porphyrin and 5,10,15,20-tetrakis(pentafluorophenyl)porphyrin. A 50 μM of PS was used under white light at an irradiance of 200 mW cm^{-2} over 20 min showing that the most effective PS caused a 4.1 log (Tetra-Py⁺-Me) and a 3.4 log (Tetra-Py⁺-C₅) reduction in the concentration of viable conidia. An increase of the porphyrin carbon chain led to a reduction of the inactivation of conidia, in both PS series [105].

From these reports, one can detach the factors that influence the efficiency of PDI of food-borne pathogens:

- irradiation time (and consequently, light dose) [53-55, 58, 80];
- the surface specificity of food, as shown by *in vivo* studies [58];
- cell-bound PS concentration as shown in bacteria [58] and in fungi [45];
- the use of powerful light sources (light emitting diodes) [103];
- the size of the *N*-alkyl chain on the porphyrin structure as shown on the photoinactivation of fungi spores, mainly by affecting their binding to cellular material [105];
- initial concentration, in the case of 5-ALA [55], and incubation time (or concentration of endogenously produced porphyrins) [53-55].

3. Applications for domestic, industrial and healthcare settings

At a time when environmental protection, energy efficiency, sustainability and entrepreneurship are guiding principles of new technological solutions, it makes sense that the collaboration between researchers and businessmen / entrepreneurs is reinforced, combining innovation and creativity. In this partnership, which is an increasingly evident reality, the design, production and commercialization of multi-functional, reusable and durable products arise as emerging opportunities. Portuguese companies as Success Gadget and Revigrés are examples of this close collaboration. The first of these companies developed silica nanoparticles with different active ingredients (anti-mosquito, antimicrobial, antifungal) with applications in the textile industry (clothing and footwear), construction (paints) and health (antifungal socks, anti chilblain gloves and socks for diabetic foot). The innovation of these materials is linked to wash off resistance (they withstand up to 80 washes) and when applied in paints, varnishes or mortar their effect lasts up to five years [146]. The second company, Revigrés, sells self-cleaning tiles suitable for outdoor use (façade cladding and external cladding), since its titanium oxide coating, when in contact with sunlight, promotes the degradation of gaseous pollutants. Also, whenever the tile is wet, a wet film formed allows the removal of dirt. These properties reduce the maintenance costs and also promote cleaning in areas of difficult access [147].

Despite these innovative examples, there is still a long way to go with regard to fast, effective and long-lasting elimination of microorganisms on surfaces, whether in the household, industrial or healthcare settings. The application of the photodynamic principle to this context seems to be a promising option, according to studies conducted in recent years. Table 3 gathers the potential non-clinical applications of porphyrin-based photoactive compounds/materials suggested by the referred literature.

Table 3. Potential non-clinical applications of porphyrin-based photoactive compounds/materials

	References
Water and environment	
Algicides	[69]
Control of fish infections in aquaculture systems	[81]
Control of insect pests around poultry-producing facilities, in barns, feedlots, manure storage areas, greenhouses	[99]
Disinfection of polluted waters from fish-farming ponds	[49, 81, 110]
Filters for water treatment	[85]
Larvicides/insecticides/pesticides	[23, 46-48, 50, 59, 64-68, 99, 104, 109]
Wastewater treatment	[36, 37, 70, 93, 96]
Water disinfection	[52, 63, 72, 96]

Food safety

Decontamination of food-related surfaces (packaging material)	[55-57, 75, 80, 101]
Decontamination of ready-to-eat fruits, frozen fruits, pastry products or similar	[58, 103]
Decontamination of sprouts	[44, 94]
Wraps or castings for fresh and processed products	[80]

Domestic, industrial and healthcare settings

Self-cleaning surfaces	[43, 63, 75-77]
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More specifically:

Hygiene products or packaging materials	[82]
Polymeric materials (syringes, IV bags and tubing, catheters, hemodialysis filters and gloves)	[61, 73, 79]
Privacy curtains in hospital rooms	[73]
Protective clothes and masks	[73]
Sources of nosocomial infections textile (bedding, surgeons gowns, pillows)	[73, 79]
Walls, floors, instruments	[88]

Other:

Hand hygiene	[74]
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910 3.1 Porphyrin-embedded fabric

911 The thought of incorporating porphyrins into different textile materials to create
912 protective clothing in the household, industrial and hospital settings, including masks, gowns,
913 caps, bedding and other textiles, was suggested nearly 10 years ago. The first antimicrobial
914 porphyrin textiles were reported by Bozja et al. [73], named as light-activated antimicrobial
915 materials. Novel materials were synthesized by the grafting of PPIX and zinc protoporphyrin IX
916 (Zn(II)-PPIX) to nylon fibers and their antimicrobial effectiveness tested against *S. aureus* and *E.*
917 *coli*. Samples of the textiles were inoculated with bacteria and irradiated with incandescent
918 light (at 10,000, 40,000, and 60,000 lx for 30 min or at 40,000 lx for 5, 15 and 30 min). At
919 40,000 lx, these fibers showed increased antimicrobial activity against *S. aureus* with increasing
920 exposure time. The samples grafted with PPIX-ethylene diamine (PPIX-ED) were able to
921 inactivate more than 95% of *S. aureus* after 30 min of irradiation upon 60,000 lx, but no
922 reduction on *E. coli* survival was observed. The samples grafted with the zinc(II) complex of
923 PPIX-ethylene diamine were able to eliminate 94% of *S. aureus* at 40,000 lx but a light intensity
924 of 60,000 lx was required to attain a reduction of 30% on *E. coli* survival. The zinc samples were
925 more effective than the corresponding free-base. With suitable changes, these materials have
926 been suggested to be used for privacy curtains in hospital rooms, laboratory coats for medical
927 personnel, or other fiber or plastic surfaces within the hospital environment considering that
928 the normal rooms' illumination is approximately 30,000 lx [73].

929 Other authors relied on the possibility of creating pioneering self-sterilizing fabrics
930 from cotton fiber, due to its resistance to repeated washing or exposure to cleaning products

[86, 87, 108]. Cotton fabrics, which essentially consist of cellulose fibers, can be easily modified by substitution of a relative small number of their numerous -OH nucleophilic groups. *Meso*-arylporphyrins bearing acetylenic units (ZnTri-Py⁺-MeCCH and Tri-Tolyl-OCCH) were grafted on cotton fabric with azide groups via "Click-Chemistry". Covalently attached porphyrin accounted for 70% of the total amount of PS and the remainder (around 30%) corresponded to porphyrins only sequestered in the cellulose fiber web. This material was impregnated with *E. coli* and *S. aureus* ($\approx 10^6$ CFU per 10 cm Petri dish) and irradiated with white light (1000 lx) for 24 h. For both bacteria, a *c.a.* 0.74 log reduction was achieved after irradiation [87]. Soon afterwards, the same group reported a modification in porphyrin immobilization envisaging further industrial applications. In this way, covalently neutral, anionic and cationic 1,3,5-triazinylporphyrin derivatives were grafted on non-modified cotton fabrics using cyanuric chloride (2,4,6-trichloro-1,3,5-triazine) as linking agent. The photodynamic activity of these new cotton fabrics was tested *in vitro* against *S. aureus* with white light for 24 h (0.16 mW cm⁻²). Sterile photosensitive textiles (3.5 × 3.5 cm), previously autoclaved for 15 min at 120 °C, impregnated with bacterial inoculum ($\approx 10^6$ CFU mL⁻¹), deposited in a sterile Petri dish, were then incubated at 37 °C for 24 h under white light irradiation in wet atmosphere. All surfaces caused a photo-bactericidal effect at a quantity as low as ≈ 10 μ mol of active compound per fabric square sample. After 24 h of light exposure, percentages of bacterial survival reduction were 37%, 93.7% and 100% for anionic, neutral and cationic surfaces respectively. The three materials did not show any photodynamic activity against Gram-negative bacteria [86, 108].

Mosinger et al. [84] produced a novel polyurethane nanofabric using the NanospiderTM electrospinning industrial technology [148] which is used for the preparation of fibers with diameters ranging from nanometers to a few microns and for materials with a high surface area and porous structure [149]. The material was doped with 5,10,15,20-tetraphenylporphyrin (TPP). The nanofabrics, composed of polymeric nanofibers had 0.03 mm thickness (2 g m⁻²) and contained 0.12% TPP. A hydrophilic variant was also produced, containing in addition 0.6% sodium dodecyl sulfate and the photodynamic function of this nanofabric variant was tested against *E. coli*. Small pieces of nanofabrics were inoculated with bacteria and illuminated with white light for 60 min. After irradiation and incubation on agar plates, no bacterial growth was observed on the nanofabric pieces. Daylight was at least as effective as artificial light. TPP incorporated in both nanofabrics variants were able to produce ¹O₂ with high efficiency. Among the exceptional properties of these nanofabrics are the large specific surface area, light weight, chemical specificities, low cost and mechanical flexibility. The same authors studied three polyurethane nanofabrics containing respectively, 0.1%

Zinc(II) phthalocyanine (ZnPc), 0.1% ZnTPP and a mixture of PS (0.1% ZnPc and 0.1% ZnTPP). Another polyurethane nanofabrics was also prepared but containing two independent layers, one doped with 0.1% ZnPc and the other doped with 0.1% ZnTPP. All nanofabrics were placed on agar plates and inoculated with a suspension of *E. coli* in an undetermined concentration, and illuminated with white light for 30 min. All PS-doped nanofabrics were able to kill bacteria on their surfaces when exposed to white light [85].

Later, Jesenska et al. [76] prepared electrospin polymeric nanofibers of polyurethane LarithaneTM (PUR), polystyrene (POS), polycaprolactone (PCL), and polyamide 6 (PA6) doped with 1% TPP. The PUR, POS, and PCL nanofiber materials were able to inhibit completely the *E. coli* growth after 60 - 90 min of irradiation with white light, while lower antibacterial activity was found for PA6 nanofiber materials. The photodynamic effect was attributed to ¹O₂ production and all tested nanofiber materials exhibit prolonged antibacterial properties after being treated with long-duration irradiation due to the formation of H₂O₂, even in the dark. The photoinactivation efficiency also depended on oxygen permeability and diffusion coefficients as on the diameter of the polymeric nanofibers. Soon after, the same group [60] selected another medical-grade nanofiber material, polyurethane Tecophilic[®] and also polycaprolactone (PCL) both loaded with TPP to study the photoinactivation of non-enveloped DNA viruses (mouse polyomaviruses, 10⁵ plaque forming units, PFU) and enveloped DNA viruses (baculoviruses pVLVP1, 10⁴ PFU). Viral suspensions were applied to the surface of small pieces of the nanofiber textiles doped with 1% TPP and were irradiated with white light for 30 min. The virus were retrieved from the surface of the textiles and used to infect 3T6 fibroblasts (for the mouse polyomavirus) and the Sf9 insect cell line (for baculovirus) [60]. Singlet oxygen released from the surface of doped polyurethane Tecophilic[®] and PCL upon irradiation efficiently inactivated both the non-enveloped and enveloped viruses and both types of polymer nanofibers are nontoxic to viruses in the absence of light. The results showed that baculovirus is more resistant to photoinactivation than the polyomavirus. The textiles were also tested after a year of storage at room temperature in the dark with the same results which indicate their long-term photovirucidal efficiency [60].

3.2 Porphyrin-embedded paper

Another possible application of microbial PDI is in hygiene products or packaging materials. Cellulose is thus envisaged as an exceptional sustainable material as it is degradable and renewable [82]. Paper or cardboard can thus be sanitized prior to use to avoid contamination. In this scope, a tricationic porphyrin, 5-(4-aminophenyl)-10,15,20-tris(1-

methyipyridinium-4-yl)porphyrin (Tri-Py⁺-Me-NH₂) was grafted on cellulose filter paper by using cyanuric chloride, without previous chemical modification of the cellulosic support, as already mentioned [108]. Sterile photosensitizing filter paper disks (0.5 cm diameter) impregnated with *E. coli* and with *S. aureus* (10⁵ CFU mL⁻¹) were deposited on sterile Petri dishes, and then incubated at 37 °C for 24 h under white light irradiation (9.5 J cm⁻²) in wet atmosphere. A strong photobactericidal effect was observed after light exposure, since no surviving bacteria were detected on grafted filter paper [82].

3.3 Porphyrins immobilized in other support materials

Krouit et al. reported several works considering the preparation of plastic materials, based on natural and synthetic porphyrins, able to inactivate Gram-positive and Gram-negative bacteria [77-79]. Photobactericidal surfaces were prepared, containing different amounts of PPIX units (0.19% to 1.1%) covalently linked to cellulose esterified with lauric acid [77]. Besides, plastic material incorporating synthetic cationic porphyrin derivatives (0.14% to 0.57% Tri-Tolyl-Py), was also prepared in order to achieve a better photobactericidal activity. In this work the cellulose was derivatized with chloroacetyl groups [78]. Based on the first work the group prepared cellulose laurate esters plastic films grafted by different carbon-spacer arms (4- or 11- carbon) to synthetic porphyrins (0.08% to 0.58%) [79]. The photodynamic activity of these polymers was tested against *E. coli* and *S. aureus*. Disks with 2 cm diameter were cut out of the porphyrinated plastic films and deposited onto nutrient agar seeded with the target strain and irradiated with visible light (1.7 mW cm⁻²) for 24 h at 37 °C. The growth of the microorganisms was examined visually under the porphyrinated disks. The cationic porphyrinic chloroacetyl cellulose chlorides, with porphyrin content higher than 0.20%, inactivated the two bacterial strains whereas films with lower porphyrin content were found more active against *S. aureus* than against *E. coli* [78]. In comparison, the films based on PPIX lauryl cellulose esters needed at least a porphyrin content value of 0.52% to photoinactivate *E. coli*. [77] These results confirmed the higher photobactericidal effect of the cationic porphyrinic plastic films [79].

The possibility of incorporating PS-modified-cellulose nanocrystals into paper, fabrics and plastics was highlighted by Feese et al. [75] who created a conjugate cellulose nanocrystals-cationic porphyrin (CNC-Por) with the aim to rapidly inactivate a range of bacteria, with minimal cost. As stated, cellulose nanocrystals have several advantages over amorphous cellulose ester: they have a particular degree of molecular control for precise functionalization due to their rigidity, defined surface, structure and dimensions; they are

readily biodegradable; they represent a renewable resource, which eases the development of new advanced materials with photobactericidal activity [75]. To test the performance of these materials, a zinc(II) complex of a cationic water-soluble ethynylphenylporphyrin (ZnTri-Py⁺-Me-CCH) was covalently attached to azide-modified cellulose nanocrystals. Saline buffered suspensions of *E. coli*, *S. aureus* and *Mycobacterium smegmatis* ($\approx 10^8$ CFU mL⁻¹) were incubated in the dark for different time intervals with 20 μ M CNC-Por which refers to the concentration of the porphyrin on CNC-Por present in the suspension. Then they were illuminated with white light (60 mW cm⁻²). Six log units reduction in viable cells were observed against *S. aureus* (5 min incubation, 30 min irradiation), 3.5 log units for *M. smegmatis* (45 min incubation, 30 min irradiation), and 2 log units for *E. coli* (60 min incubation, 30 min irradiation) [75].

The PDI of bacteria mediated by these same conjugates (20 μ M) was further tested as a function of bacterial strain, incubation time and illumination time on *Acinetobacter baumannii*, multidrug resistant *A. baumannii* (MDRAB), methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa* ($\approx 10^8$ CFU mL⁻¹) with visible light (65 mW cm⁻²) [43]. A reduction of 6 log units in viable cells was observed for MRSA (5 min incubation), 5 – 6 log units for *A. baumannii* (30 min incubation) and MDRAB (15 min incubation), and 2.5 log units for *P. aeruginosa* (60 min incubation) at a total light dose of 118 J cm⁻² (30 min irradiation). Confocal laser scanning fluorescence microscopy of *A. baumannii* and *S. aureus* incubated with CNC-Por suggested a lack of internalization of the PS, supporting the hypothesis that direct binding and uptake of the PS are not necessary requirements for PDI by CNC-Por. On the other hand, the ZnTri-Py⁺-Me-CCH, when in solution and not bound to the cellulose nanocrystals, led to the fluorescence being localized on *A. baumannii*. Even though it was not determined whether the cationic PS was internalized by the bacterium, or simply bound to the bacterial cell membrane. So the production of ¹O₂ and other radical species in close proximity to the bacteria may be sufficient to result in cell inactivation or death. Despite forming an insoluble suspension, CNC-Por conjugate exhibited excellent antimicrobial activity for all strains examined with the exception of *P. aeruginosa*, which was only moderately inactivated [43].

The efficient photodynamic effect of other photoactive surfaces was demonstrated by Funes et al. [63] The authors used two polymeric porphyrinated films formed by electrochemical polymerization of free-base 5,10,15,20-tetrakis(4-(*N,N*-diphenyl)aminophenyl)porphyrin (Tetra-*N,N*-DiPh-film) and its complex with Pd(II) (Pd(II)Tetra-*N,N*-DiPh-film) on optically transparent indium tin oxide (ITO) electrodes on microbial cell suspensions. The polymeric surfaces (0.7 × 3.0 × 2.1 cm²) were placed inside of *E. coli* and

Candida albicans suspensions ($\approx 10^6$ CFU mL⁻¹) and irradiated with visible light (90 mW cm⁻²) for different time periods. The viability of microbial cells irradiated with visible light depended on the light exposure. Both Tetra-*N,N*-DiPh- and Pd(II)Tetra-*N,N*-DiPh-films exhibited a photosensitizing activity causing a ≈ 3 log decrease of *E. coli* survival after 30 min of irradiation and a ≈ 2 log decrease in *C. albicans* viability. The advantages of these electrodes are that they represent an appropriated surface to obtain mechanically stable electropolymeric films, they are optically transparent to visible light, and also that they can be easily and quickly removed from the media after cell inactivation, avoiding permanent photodynamic effects [63].

In another approach to photoinactivate *C. albicans* [61], novel photoactive flexible bridged polysilsesquioxane thin plastic films (SSO-PS) doped with 5-(4-carboxyphenyl)-10,15,20-tris(4-methylphenyl)porphyrin (Tri-Tolyl-CO₂H) were evaluated in aqueous suspensions (with 5 μ M of Tri-Tolyl-CO₂H) and on two SSO-PS films (with 2.6×10^{-4} w/w and 5.2×10^{-4} w/w concentration of Tri-Tolyl-CO₂H in each film). The yeasts treated with 5 μ M of Tri-Tolyl-CO₂H were incubated 30 min in dark at 37 °C later irradiated with visible light (90 mW cm⁻²). In the case of polymeric films, their surfaces ($0.7 \times 2.5 = 1.75$ cm²) were inoculated with yeast suspension ($\approx 10^6$ CFU mL⁻¹), irradiated, and then placed in saline buffer for serial diluting, plating and colony counting. With 5 M of Tri-Tolyl-CO₂H (non-cationic porphyrin), an expected small photoinactivation effect (0.5 log decrease) was observed after 60 min. The viability of *C. albicans* cells in irradiated SSO-PS films depended on the light exposure although no differences were observed between the two SSO-PS films. They caused a photosensitizing activity of ≈ 2.5 log decrease of *C. albicans* survival after 60 min of irradiation (324 J cm⁻²) [61]. Comparatively to the results obtained by Funes et al. [63], the main difference of the present SSO-PS films is their versatility as plastic flexible materials at room temperature, easy to obtain and ability to shape the surfaces. They also keep a higher antifungal activity against *C. albicans*.

Microporous silica gels prepared by sol-gel process from tetrakis(2-hydroxyethoxy)silane (THEOS) have been used to immobilize the cationic Tetra-Py⁺-Me [88]. THEOS was used to ensure toughness and greater stability of silica-Tetra-Py⁺-Me composites. The antimicrobial activity of these composites was compared with their tetramethoxysilane (TMOS) analogs against *E. coli*. Nutrient agar inoculated with *E. coli* (10^6 CFU mL⁻¹) was poured over the composites at the bottom of plastic plates and illuminated with visible light (10,000 lx for 0, 1.5, and 3 h, corresponding to a light dose of 0, 7.9 and 15.8 J cm⁻², respectively). Growth of *E. coli* in nutrient agar decreased with increasing light energy for all the composites. On the dense THEOS composites, the 15.8 J cm⁻² light energy led to the total growth inhibition (6 log). In the case of TMOS, the higher bactericidal effect was established for the weak composites (6

log reduction with the composite with PEG). In general, THEOS composites with the lower specific surface areas were more effective than TMOS analogs. The antimicrobial activity of these composites was mainly influenced by total Tetra-Py⁺-Me content in the bulk, specific surface areas, and by the thicknesses of the composites. Among THEOS composites, their flexibility brought about by PEG 600, improved oxygen diffusion. All the composites showed good adhesion to glass, and THEOS ones have not shrunk at least for 3 months. These properties, along with a high shape stability of THEOS-Tetra-Py⁺-Me composites, make them favorable photosensitive materials [88].

The same microbiological method was applied to evaluate the antibacterial photodynamic effect of mesoporous organosilica – porphyrin composites obtained by entrapment of TPP and Tetra-Py⁺-Me into three polysilsesquioxanes prepared by the sol – gel method from 1,2-bis(triethoxysilyl)ethane (BTE), 1,6-bis(triethoxysilyl)hexane (BTH), and 1,8-bis(triethoxysilyl)octane. The Si-matrices of the porphyrin composites under study are biocompatible and unresponsive to photosensitization. The pre-polymer-porphyrin mixtures were polymerized in a glass Petri dish and were inoculated with *E. coli* (10⁵ CFU mL⁻¹) immobilized in nutrient agar. Irradiation was performed with visible light (with 5,500 lx for 0 h, 1.5 h, and 3 h corresponding to 0, 4.3, and 8.7 J cm⁻², respectively). The fluorescence and antibacterial effects of the TPP composites increased with increasing incident light energy and were dependent on the mesoporosity of the matrix. The composites with the higher mesoporosity are preferable since the matrix controls both oxygen diffusion through the material and quenching of the excited PS. For that reason, TPP – BTH polymer exerted the highest biocidal effect (≈ 5 log decrease with 8.7 J cm⁻²) and the other TPP composites reduced cell viability to less than 1 log. Tetra-Py⁺-Me – BTE polymer was less effective. The complete inactivation of the bacteria attained with TPP – BTH demonstrates that the photodynamic process takes place in the whole volume of the agar layer (1000 μm thick) and not only on its contact surface with the TPP – BTH film [89].

In vitro preliminary antimicrobial tests with palladium(II) complex of 5,10,15,20-tetrakis(4-carboxyphenyl)porphyrin)(Pd(II)TetraTPPCO₂H) – Zn₂Al/PU (polyurethane) composite films (0.4 wt% of Pd(II)TetraTPPCO₂H for 1% of Zn₂Al filler loading) were able to totally inhibit two strains of *S. aureus* and *P. aeruginosa* seeded on nutrient agar (≈ 10⁵ CFU mL⁻¹) and incubated 24 h at 37 °C under continuous white light illumination at an irradiance of 100 W cm⁻². Considering the low amount of PS, the Pd(II)TetraTPPCO₂H – Zn₂Al/PU film proved to be a highly efficient light activated antimicrobial surface [83].

According to Eicshner et al. [74], antimicrobial photodynamic treatment might become acceptable as a tool for hand hygiene procedures and also in other skin areas, as washing of the hands is of little use if, for example, a hospital environment is heavily contaminated [150]. So, the possibility of rapidly and efficiently inactivating MRSA, enterohemorrhagic *E. coli* (EHEC) and *C. albicans*, using the photodynamic approach, was revealed *in vitro* with different concentrations of Tetra-Py⁺-Me illuminated with visible light (50 mW cm⁻²) for 10 s and 60 s. A light dose of 0.5 J cm⁻² and 1 μM of Tetra-Py⁺-Me achieved a PDI of ≥ 3 log of MRSA and EHEC. Incubation with higher concentrations (up to 100 μM) of Tetra-Py⁺-Me caused bacterial inactivation by more than 5 log. Efficient *C. albicans* inactivation (≥ 5 log) was achieved at a light dose of 12 J cm⁻². Since Tetra-Py⁺-Me has no clinical approval to be used as a medical drug, it was suggested that similar porphyrin molecules with similar properties that can be approved for use in humans should be developed in the near future. This rapid inactivation procedure may be useful for clinical, on industrial purposes, but it is also feasible in environmental technology. Processes with short application times, simplicity, efficacy and low costs are desirable and needed [74].

In addition to testing the PDI of microorganisms, most of these studies tried to obtain a mechanistic explanation of the action of the PS immobilized on these supports, mainly based on the concept of 'photobactericidal surface', introduced by Midden and Wang who demonstrated that a light-induced flow of ¹O₂ on the material surface was responsible for cell inactivation [151]. The scientific literature has disclosed distinctive forms of interaction between porphyrins and bacterial cells. If on one hand it has been argued that porphyrins can penetrate the cell wall to the periplasmic space and bind to the cytoplasmic membrane [152-154]; on the other it has also been demonstrated that they can have a dual location (bound to the cell wall and to the nucleic acids) [155]; or do not have to penetrate [156, 157] or even come into contact with the cells [158].

When porphyrin molecules are immobilized in solid supports, their availability to bind and eventually penetrate into cells is reduced. Thus, to produce the same inactivation results, the concentration used in the supports must be higher than that which would be used in solution [75]. This fact supports the idea that direct binding and uptake of the PS are not necessary requirements for PDI [75]. This was justified by the production of ROS on the surface of the material, mainly ¹O₂ during activation of the light-porphyrinated materials followed by their dissemination and possible interaction with the target cell [77, 78]. To interact against the cells and lead to cell death, these species would have to be longer-lived, and/or could be generated in close proximity to the bacteria [43, 75, 83].

4. Conclusions

Although not yet established as an antimicrobial therapeutic procedure, the photodynamic inactivation of microbes and other organisms within the environmental context continues being improved and increasingly optimized to meet the urgent need for alternative options to commonly used antimicrobials to avoid development of microbial resistance.

Among the main advantages of this approach are its nature of multi-target cellular damage and the possible use of a powerful and free light source, as sunlight. In the 1990s, when it was found that a photosensitizer with positive charges on its structure allowed the efficient inactivation of cells with complex structural features, such as Gram-negative bacteria, the research in photodynamic inactivation took a new breath.

In addition to being remarkably effective against a multitude of micro(organisms), the use of photosensitizers such as porphyrin derivatives, allows for the designing of an immense diversity of structurally different molecules that can be made more efficient and, in the non-clinical framework, also to be harmless to the environment and to humans and animals, if the photosensitizer molecules are immobilized on inert solid supports.

Likewise, the use of porphyrin concentrations in the micromolar range or from natural sources enables photodynamic inactivation to be used exclusively for its intended purpose without further impact.

The diversity of possible applications that have been proposed in recent years, as well as the progress made in this research area demonstrate that PDI is a promising method of disinfection/sterilization with promising practical application in the short term.

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