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Photoinduced antibacterial activity of two dicationic 5,15-diarylporphyrins

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ABSTRACT

Antimicrobial photodynamic treatment combines the use of photosensitizers (PSs) and visible light to kill bacterial cells. Cationic porphyrins are PSs largely used against bacteria and, among them, those featuring one positive charge on each of the 5,10,15,20-tetraaryl substituent (tetracationic) are the most used. The aim of this study was to synthesize two dicationic 5,15-di(N-alkyl-4-pyridyl)porphyrins, bearing methyl (PS **3**) and benzyl (PS **4**) N-alkylating groups, and to compare the efficiency in antibacterial photodynamic treatment, upon irradiation with a halogen-tungsten white lamp.

The killing efficiency of the PS **4** was constantly found higher than that of the PS **3** against both pure and mixed cultures of laboratory model microorganisms as well as against wild wastewater microflora. The two PSs are comparable as regards singlet oxygen generation, but show a different repartition coefficient; the more lipophilic benzylated PS **4** shows a better interaction with the bacterial cells than the methylated one (PS **3**). The data support the hypothesis that an efficient PS–cell binding is required to obtain significant effects. A correlation among cell binding, photoinactivation and PS lipophilicity is suggested.

1. Introduction

The control of microorganism growth is a relevant topic in several contexts and can be achieved by means of chemical or physical agents or by their simultaneous combination. A disinfection system based on the photodynamic approach could potentially be applied to distinct needs. This approach requires the concomitant application of a chemical compound, the photosensitizer (PS), and of a visible light characterized by a suitable energy and wavelength of emission able to excite the PS. The PS, bound to the target cells and excited by light irradiation, transfers the adsorbed energy to any molecule present in the close proximity (<0.02 mm) [1]. When this energy transfer process involves molecular oxygen, singlet oxygen ($^1O_2$) and/or reactive oxygen species (ROS) become the final products. Both these oxidizing species react with most biological molecules thus inducing damages to most of the cell structures finally leading to cell death [2]. The PSs used to this end are substantially dyes which share, as common feature, the presence of a large number of conjugated double bonds thus allowing the interaction of the π electrons with low energy radiations i.e. the visible light.

Among the several classes of known PSs, encompassing both natural and synthetic compounds, cyclic tetrapyrrolic derivatives, including porphyrins and the reduced congeners (chlorines and bacteriochlorines), benzoporphyrins and their aza analogoues phthalocyanines, have been widely studied as they constitute a particularly versatile basic frame to design new PSs [3]. Such versatility accounts for the possible synthesis of differently structured molecules on the base of the specific characteristics needed for cell interactions, as prokaryotes and eukaryotes feature very different envelopes. Actually various PSs can be used for the photoinactivation of prokaryotes, however the efficacy of a given PS can dramatically differ when the target is a Gram positive or Gram negative bacterium [4]. Indeed, it is well known that Gram negative bacteria are more resistant to the photodynamic action than Gram positive. This has been ascribed to the presence of the structurally complex outer membrane the Gram negative are endowed with [5]. To improve the efficiency of PS against Gram negative two features are thought to be important: (1) the presence of positive charges on the PS, that promote a tight interaction with the negative charges of the LPS of the outer membrane [6], and (2) a degree of lipofilicity giving a $\log P > 0.5$ thus favouring the interaction with the outer membrane [7].

Porphyrins display peculiar PS structural characteristics that can be easily achieved tuning the reagents used in their synthesis, which is generally based on an acid catalyzed condensation of

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aromatic aldehydes with pyrrole. Furthermore, as all the porphyrins are characterized by a wide-range absorption spectrum extended from the blue Soret band to the green and red Q bands, these PSs might be used in the presence of broad band emitting light sources, including sunlight. This characteristic of the porphyrin skeleton makes these PSs also suitable for the treatment of different matrixes contaminated by bacteria. As an example these PS could be used for the disinfection of microbiologically polluted waters [8,9].

Tetracationic tetraarylporphyrins, such as the commercial tetra(N-methyl-4-pyridyl)porphyrin tetratosylated and the tetra(4-trimethylammonium-phenyl)porphyrin tetraiodide and some of their congeners, have been widely studied in the photodynamic antimicrobial chemotherapy (PACT) [10–12]. From these studies the presence of four ammonium groups (one for each *meso* aromatic moiety) appears to be a mandatory requirement to achieve an efficient antimicrobial activity, although some activity has been also reported for three-cationic tetraarylporphyrins [13]. The 5,15-diarylporphyrins featuring two positive charges are expected to be less hydrophilic than the tetracationic congeners and, to the best of our knowledge, no studies on the efficacy of such compounds against bacterial cells has been yet reported, although recently *in vitro* anticancer PDT studies have shown a greater efficacy of the diarylporphyrins with respect to the tetraaryl congeners [14].

Here, the comparison of two dicationic 5,15-diarylporphyrins in the photoinactivation of representative Gram positive and Gram negative bacteria is reported. The disinfection potential of the most efficient dicationic porphyrin was also investigated, using sun light as light source, on small volumes of secondary treated wastewater samples, regarded as model of a matrix highly contaminated by a complex bacterial community.

2. Materials and methods

2.1. General

UV–vis absorption spectra were measured on a Varian Cary 50 Scan instrument. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer in $CDCl_3$ or [d_6] DMSO; chemical shifts are expressed in ppm relative to chloroform (7.28) and are reported as s (singlet), d (doublet), t (triplet), m (multiplet), bs (broad singlet). Elemental analyses were performed on a ThermoQuest NA 2100, C, H, N analyzer, equipped with an electronic mass flow control and thermal conductivity detector. Fluka F254 silica gel RP-18 (0.2 mm thick) were used for analytical thin-layer chromatography (TLC). Silica gel 60 (70–230 mesh, Merck) was used for column chromatography. Benzyl chloride and methyl iodide were commercial products (Sigma–Aldrich) and used as received. The fluence rate (irradiance, W/m^2 or mW/cm^2) was determined with a LI-COR 1800 spectroradiometer. The illuminance of sunlight was measured with a Lux meter LX-101.

The compounds 5-(4-pyridyl)-dipyrrolylmethane (**1**) and 5,15-Dipyridylporphyrin (**2**) were synthesized according to the method described by Gryko and Lindsey [15] and Goncalves et al. [16], respectively.

2.2. Synthesis of the cationic photosensitizers **3** and **4**

2.2.1. 5,15-Di(N-methyl-4-pyridyl)porphyrin (**3**)

To 20 mg (0.043 mmol) of **2** were added 10 mL of CH_3I and the solution was kept under stirring and reflux for 48 h, following the reaction outcome by TLC (RP-C18, H_2O/2-propanol/acetic acid: 4/4/2). The desired product was collected by filtration after precipitation induced by the addiction of 30 mL of Et_2O to the reaction mixture and on cooling. The solid was thoroughly washed with diethyl ether and treated with a few mL of water; the insoluble material was eliminated by filtration and 12 mg (38%) of the desired product were recovered after lyophilisation. $C_{32}H_{26}N_6I_2$, M_w = 748.4. UV–vis(H_2O): 408 nm (ε = 24,400), 506 nm (ε = 1914), 552 nm (ε = 1806), 632 nm (ε = 1004). ^{1}H NMR (D_2O) δ: −3.33 (s, 2H); 4.73 (s, 6H); 9.07 (d, 4H); 9.24 (d, 4H); 9.48 (d, 4H); 9.85 (d, 4H); 10.84 (s, 2H). Anal. Calc.: C, 51.35%; H, 3.50%; N, 11.23% Found; 51.74%; H, 3.48%; N, 11.29%.

2.2.2. 5,15-Di(N-benzyl-4-pyridyl)porphyrin (**4**)

Compound **2** (20 mg, 0.043 mmol) was treated with 15 mL of benzylchloride. The solution was kept under reflux for 48 h, following the reaction outcome by TLC (RP-C18, H_2O/2-propanol/acetic acid 4/4/2). The reaction work up, carried out as described above, yielded 15 mg (45%) of the desired product. $C_{44}H_{34}N_6Cl_2$, M_w = 717.70. UV–vis(H_2O): 410 nm (ε = 51,600), 508 nm (ε = 3512), 550 nm (ε = 3409), 631 nm (ε = 1919). ^{1}H NMR (D_2O) δ: −3.30 (s, 2H); 6.30 (s, 4H); 7.60 (m, 10H); 9.14 (d, 4H); 9.31 (d, 4H); 9.75 (d, 4H); 9.84 (d, 4H); 10.84 (s, 2H). Anal. Calc.: C, 73.64%; H, 4.77%; N, 11.71% Found; 74.01%; H, 4.81%; N, 11.63%.

2.3. Partition coefficient measurements

1-Octanol/water partition coefficient (P) was determined at 25 °C mixing equal volumes of pre-equilibrated water (milliQ, 3 mL) and 1-octanol (3 mL) containing a suitable amount of porphyrin. Typically, an aqueous solution of cationic porphyrin (≈40 μM) was stirred for 8 h in the thermostat in the presence of octanol, then aliquots of 200 μL of both aqueous and organic phases were diluted with 1.8 mL of DMF (2 mL of total volume) and the porphyrin final concentration was determined by absorption spectroscopy [17]. Values are expressed as $\log P = \log([\text{porphyrin}]_o/[\text{porphyrin}]_w)$.

2.4. Photobleaching measurements

A 15 mL solution 50 μM of porphyrin in 0.1 M phosphate-buffer (PBS, pH 7.4) was exposed to a 500 W halogen-tungsten lamp for 2 h, with a light irradiance medium value of 0.12 mW/cm^2 nm in the range 380–780 nm. The heat of the lamp was cut off by means of an aqueous filter positioned between the light source and the PS solution thus maintaining the temperature at 37 °C. Samples of 0.4 mL were collected every 15 min, diluted with 1.6 mL of PBS and the concentration of the residual PS was spectroscopically measured with the UV–vis instrument The results are reported as percentage of the absorbance measured at specific interval with respect to the value of the initial concentration.

2.5. Comparative singlet oxygen generation measurements

An aerated isopropanol solution containing 50 μM of 1,3-diphenylisobenzofuran (DPBF) and 1 μM of photosensitizer was prepared and kept in the dark. A 2 mL sample of this solution was transferred into a cuvette and irradiated from the open top side with green LED (maximum light irradiance 0.125 mW/cm^2 nm at 524 nm) at room temperature up to a maximum of 30 min. At intervals, the absorbance at 410 nm was measured. The rate of singlet oxygen production was determined from the reduction in intensity of absorbance recorded over time. A sample of DPBF-isopropanol solution was used as the negative control. In this experiments green LED lamp was used for the irradiation as DPBF, the singlet oxygen scavenger, is very unstable and undergoes self photobleaching when irradiated with blue LED and white halogen lamps. The relative singlet oxygen generation rates for PS **3** and PS **4** were determined by using Rose Bengal as a reference PS [18].

2.6. Bacterial strains and culture conditions

The microorganisms used in this study are opportunistic pathogens representative of Gram negative and Gram positive bacteria. *Escherichia coli* C1a [19], *Enterococcus faecalis* ATCC 29212 [20], *Pseudomonas aeruginosa* PAO1 [21] and *Staphylococcus aureus* ATCC 25293 (MSSA) [22] are wild type bacteria commonly used as model or control strains. *E. coli* and *E. faecalis* are also regarded as indicators of faecal contamination. Bacteria were grown overnight in Luria Bertani (LB) broth [23] under aerobic conditions at 37 °C and then diluted in M9 minimal medium [24] supplemented with 5 mM glucose to obtain a cell concentration of approx. 10^7 CFU/mL. Viable counts (expressed as colony forming units per mL, CFU/mL) were estimated by plate count technique: a standard volume (0.1 mL) of undiluted or serially diluted samples was plated on rich or selective agar medium. LB Agar plates and PCA (Plate Count Agar) plates were incubated for 24 h at 37 °C to evaluate the viable cells in pure cultures and the total heterotrophic bacteria in wastewater samples, respectively. Plates of mFC Agar supplemented with 1% rosolic acid (Difco Laboratories) were incubated for 24 h at 37 °C and characteristic blue colonies were counted for faecal coliforms. Plates of KF Agar supplemented with TTC 1% (Difco Laboratories) were incubated for 48 h at 37 °C and characteristic reddish colonies were counted for faecal enterococci.

2.7. Wastewater samples

Effluent from a conventional activated sludge plant of a Northern Italy little town, was collected after the sedimentation and used in the photoinactivation experiments. Three wastewater samples were collected in three different days on January/February 2011. The concentrations of faecal coliforms, faecal enterococci and heterotrophic bacteria were determined as described above.

2.8. Photoinactivation assays

Aliquots of PS aqueous solutions were added to pure or mixed cultures or to wastewater samples, affording the desired PS concentrations (1, 5 or 10 μM). After 1 h of static incubation at 37 °C in the dark, the cultures were irradiated with a 500 W halogen-tungsten lamp (fluence rate 48 mW/cm^2, considering the 400 nm of the whole width of the lamp emission spectrum) for 75 min (energy density 216 J/cm^2). The lamp was placed at a distance of 20 cm above the sample and a 1.5 cm thick circulating water/glass filter was interposed to avoid overheating. Alternatively, the samples were irradiated by sunlight (with or without a UV filter); in these cases lux values and air temperature (°C) were measured. Survivors were quantified using the viable count technique as describe above (limit of detection, 10 CFU/mL). A panel of controls was set for each experiment: PS untreated and dark incubated samples (−PS, −light), PS treated and dark incubated samples (+PS, −light), PS untreated and irradiated samples (−PS, +light).

2.9. Porphyrin cell binding assays

Bacterial cultures (10^7 CFU/mL) in M9 medium supplemented with 5 mM glucose, were incubated at 37 °C in the dark for 1 h in the presence of 10 μM PS. After this period, the samples were centrifuged (13,000 rpm for 10 min) and the supernatants were spectrophotometrically analyzed to determine the concentration of the unbound PS: the data were registered in the blue wavelength range at 410 and 408 nm, respectively representing the λ_{max} of absorbance for PS **4** and PS **3** in the aqueous phase. The pellets were suspended in 500 μL of SDS 2% and incubated at room temperature for 30 min; the samples were then centrifuged (13,000 rpm for 10 min) and the supernatants were analyzed as above measuring the absorbance of PS **4** and PS **3** that, in SDS solution, are at 420 nm and 418 nm, respectively. When necessary, the cell pellets were further treated with HCl 0.1 M. The porphyrin concentrations were obtained interpolating the data on a calibration plot in the range 1–10 μM. The experiments were performed in triplicate.

2.10. Electron microscopy

Aliquots of pure culture of *E. coli* and *E. faecalis* from photoinactivation experiments, were processed for electron microscopy observations, according to Tettamanti [25]. Briefly, after fixation in 4% glutaraldehyde for 1 h at room temperature, cells were post-fixed in 1% osmic acid for 20 min at room temperature. After dehydration in an ethanol series, they were embedded in an Epon/Araldite 812 mixture. Thin sections, stained with uranyl acetate and lead citrate, were observed with a Jeol 1010 EX electron microscope. Images were acquired with an Olympus Morada TEM CCD camera.

2.11. Statistical methods

The photoinactivation experiments were repeated at least 3 times on separate dates. Mean and standard deviation calculations were performed using Microsoft Excel 2003 (Microsoft Corporation, Redmond, WA). Data were analyzed by means of one-way ANOVA (Origin® 7.0 SR0). Significant effects of treatments ($p < 0.05$) were estimated with the following contrasts: −PS +light vs −PS −light; +PS −light vs −PS −L; +PS +light vs −PS −light.

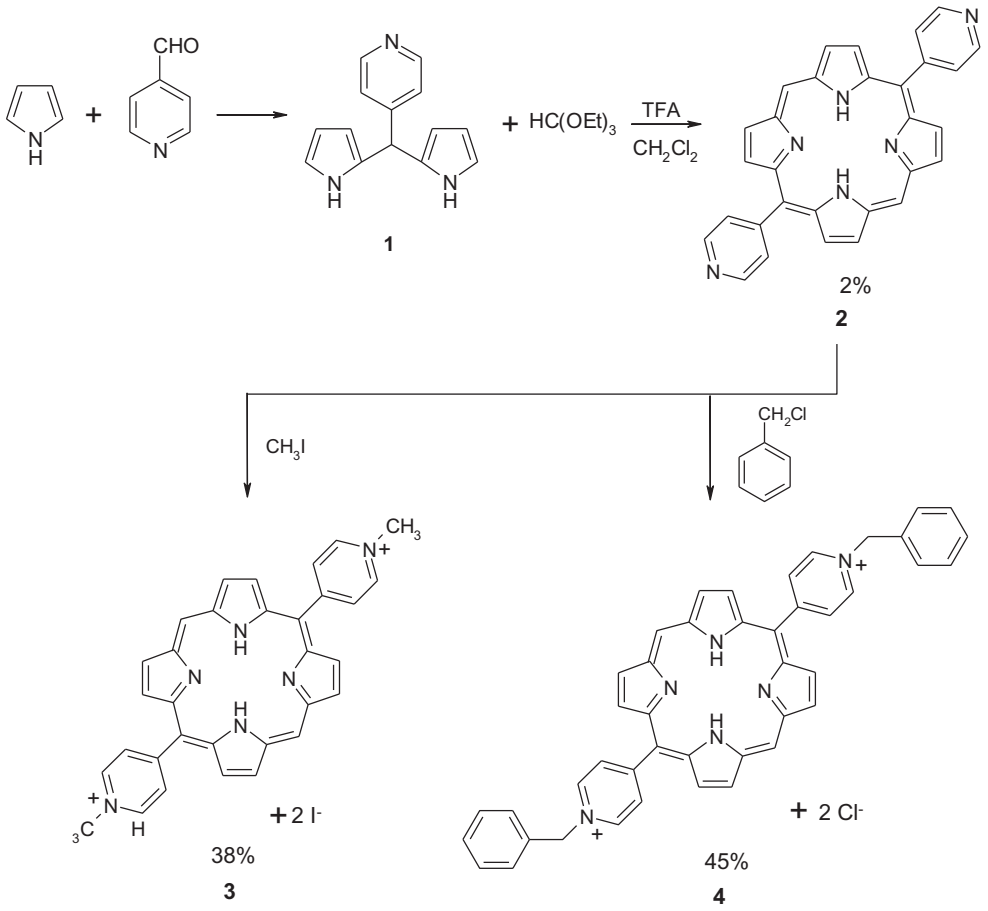
3. Results

3.1. Synthesis and physical properties of diarylporphyrins

The synthetic pathway proposed by Boyle [26] was chosen to synthesise the parent compound 5,15-di(4-pyridyl)porphyrin (**2**), from which the two cationic PSs, 5,15-di(N-methyl-4-pyridyl)porphyrin (**3**) and 5,15-di(N-benzyl-4-pyridyl)porphyrin (**4**), were obtained following N-alkylation. Accordingly, 4-pyridylcarbaldehyde was reacted with a large excess of pyrrole affording the 5-(4-pyridyl)-dipyrromethane (**1**) in a good isolated yield (60%) [15]. Compound **1** was then reacted with triethylorthoformate in CH_2Cl_2 in the presence of trifluoroacetic acid followed by air oxidation of porphyrinogen to porphyrin [16]. The chromatographic purification of the raw material yielded only 2% of the 5,15-di(4-pyridyl)porphyrin (**2**) (Scheme 1). The 5,15-di(N-methyl-4-pyridyl)porphyrin (**3**) and the 5,15-di(N-benzyl-4-pyridyl)porphyrin (**4**), both featuring positively charged pyridinium groups on the molecular frame, were respectively obtained *via* alkylation of the pyridyl nitrogens refluxing the porphyrin **2** with methyliodide or benzylchloride used as solvents. The desired compounds **3** and **4** were isolated after the formation of a precipitate caused by the addition of diethylether (Scheme 1).

The octanol–water partition coefficient (logP) values for the benzyl derivative **4** and the methyl derivative **3** were of 0.92 and 0.18, respectively. These data clear indicate a higher lipophilic character of the compound **4** with respect to **3.**

The photobleaching of compounds **3** and **4** was measured following the decrease of their Soret absorption band following exposure to the white light of the 500 W halogen-tungsten lamp. No significant differences were observed comparing the two PSs, as both porphyrins maintained approximately 85% of the intensity of the initial Soret band after 2 h of light exposure (Fig. 1). These data ensure that no severe photoinduced degradation of the porphyrin frame occurs in the time course of bacteria photoinactivation assays.



Scheme 1. Synthesis of the two cationic diarylporhpyrins **3** and **4**.

As singlet oxygen is known to be the most important agent that mediates photocytotoxicity, the two diarylporphyrins were tested for the production of this reactive oxygen species following irradiation with a green LED device. With respect to Rose Bengal, the dye used as reference compound, PS **3** and PS **4** showed a quite low production of singlet oxygen, 2×10^{-3} and 1×10^{-3}, respectively.

3.2. Photoinactivation experiments

The diarylporphyrins **3** and **4** were used to photoinactivate pure cultures of the model microorganisms *E. coli* C1a, a Gram negative bacterium, and *E. faecalis* ATCC51298, a Gram positive one. The photoinactivation experiments were performed using a bacterial density of 10^7 CFU/mL and the irradiation was carried out in a static model at room temperature under artificial white light (216 J/cm^2); the viability of *E. coli* and *E. faecalis* was monitored using the plate count method as described above. *E. faecalis* was sensitive to photoinactivation induced by both PSs; the concentrations causing a viability decrease up to the detection limit were 5 μM and 10 μM for PS **4** and PS **3**, respectively (Fig. 2). *E. coli* was completely insensitive to the treatment with PS **3**, whereas the benzylated PS **4** caused a decrease of viable cells up to the detection limit at a concentration of 5 μM (Fig. 2). Both *E. coli* and *E. faecalis* were not affected by irradiation in the absence of the PS (light control) or by the contact with any of the PSs tested in the absence of light (dark control) (Fig. S1 in Supplementary data).

The photokilling efficacy of the two PSs against *S. aureus*, a Gram-positive pathogen, and against *P. aeruginosa*, a Gram-negative opportunistic pathogen known to be more resistant to the photodynamic treatment than the other organisms used in this study [27,28], was also evaluated. As observed in the case of *E. coli*, PS **3** was totally inactive against *P. aeruginosa*, whereas 10 μM PS **4** caused a 4 log unit decrease of viable cells. 1 μM PS **4** caused the decrease of *S. aureus* viable cells to the detection limit, whereas a 5 log unit decrease was observed by 5 μM PS **3** (Fig. 3). Contrarily to what observed in *E. faecalis* cultures, increasing the PS **3** concentration did not improve the killing efficiency.

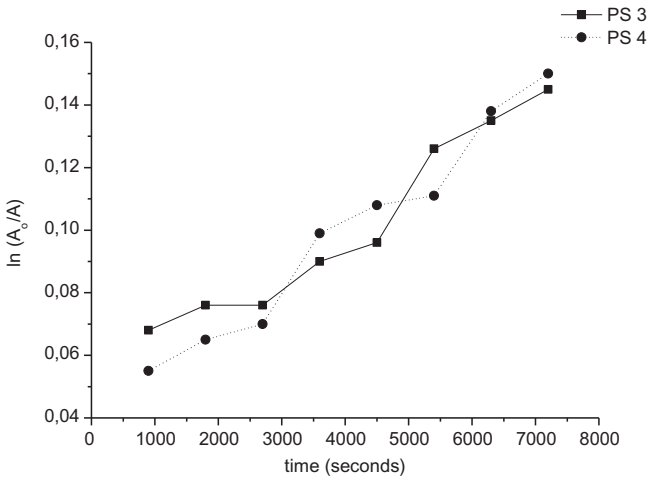


Fig. 1. Photobleaching of PS **3** (solid line) and PS **4** (dotted line) during 2 h irradiation with a 500 W halogen-tungsten lamp.

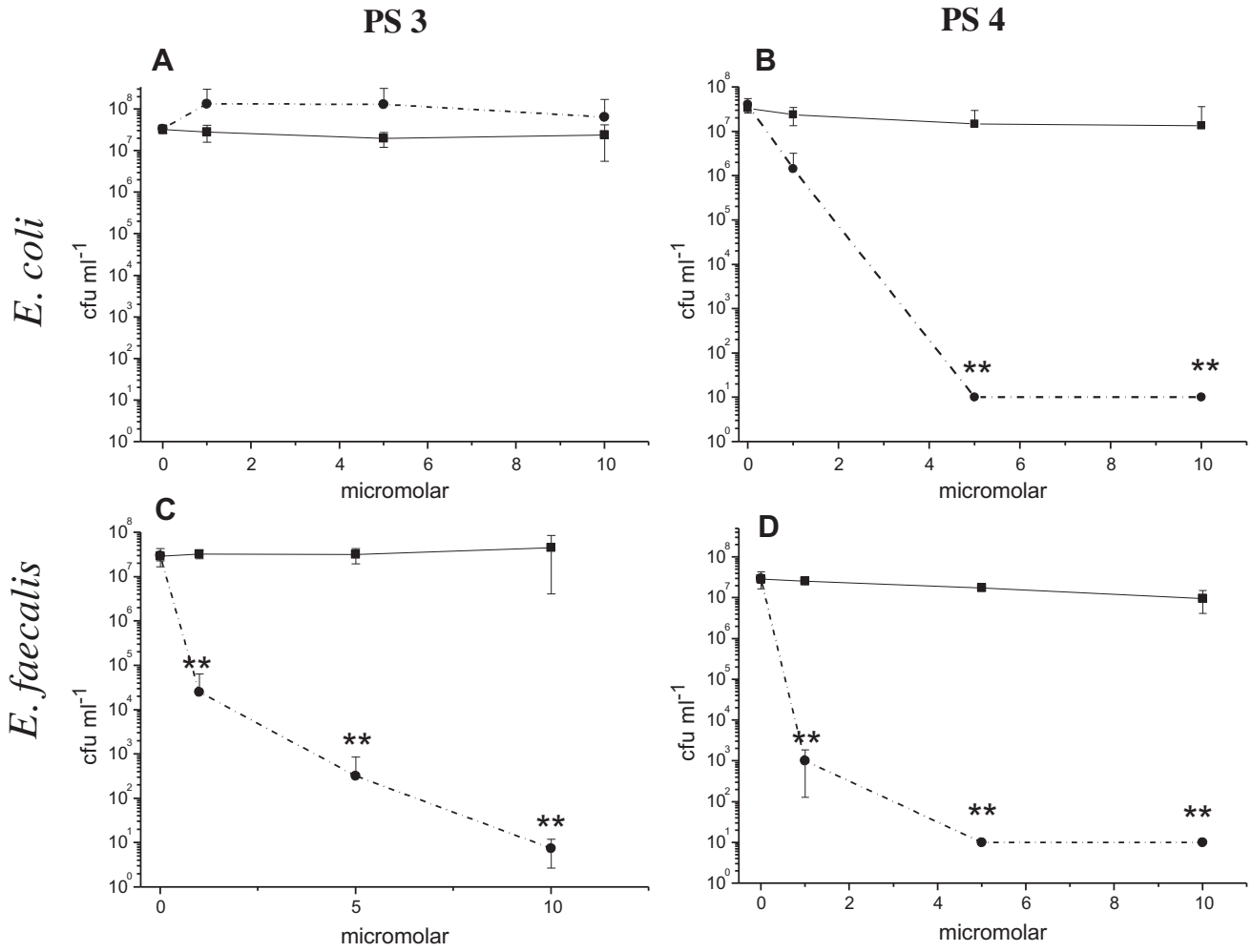


Fig. 2. Effect of increasing concentrations of PS **3** (A and C) and PS **4** (B and D) on the photodynamic inactivation of pure cultures of *E. coli* C1a (A and B) and *E. faecalis* ATCC 51298 (C and D). Cell viability was checked after 1 h dark incubation with the indicated PS (solid lines) or after 1 h of dark incubation followed by 75 min of irradiation with halogen lamp (dotted lines). The data are the mean of three independent experiments ± SD. *p* values were calculated by means of ANOVA test. ** $p < 0.01$ for + PS + light vs – PS – light samples.

3.3. Photosensitizer binding experiments

In order to evaluate whether the different efficacy of the two PSs could be at least partially ascribable to a different binding to the model microorganisms *E. coli* and *E. faecalis*, up-take studies were performed. The methylated PS **3** was less prone to interact with bacterial cells than the benzylated one (**4**) (Fig. 4). In the *E. coli* suspensions exposed for 60 min to 10 μM PS **3**, 23.33 ± 7.57% of the PS was found associated to the cells whereas 57.23 ± 9.01% was found in the supernatant. When PS **4** was used, the amount of PS associated to the cells was the 94.20 ± 18.20%, and no residual PS **4** was detected in the supernatant. In *E. faecalis*, the amount of PS **3** associated to cells was 55.33 ± 10.10% whereas the unbound fraction was 35.30 ± 6.67%. In analogy to what observed with *E. coli* no PS **4** could be detected in the supernatant. Following SDS treatment, it was possible to recover from *E. faecalis* cells the 53.40 ± 6.70% of PS **4**; the remaining PS could be recovered only with a further extraction with HCl 0.1 M (54.00 ± 4.00%).

3.4. Induction of morphological changes

Due to its higher efficiency in binding and in inducing lethality on both model organisms, PS **4** was chosen to investigate the changes induced to bacterial cell morphology following irradiation. *E. coli* or *E. faecalis* cell suspensions treated in the dark for 60 min with PS **4** (5 μM) were irradiated with an energy dose (28.08 J/cm^2) low enough to avoid cell lysis and then analyzed by transmission electron microscopy (TEM).

As it can be observed from the representative TEM micrographs, after dark incubation with PS **4** (Fig. 5B), the morphology of the outer envelope of *E. coli* cells was comparable to that of the untreated and not irradiated control (Fig. 5A). The exposure to the PS in the absence of irradiation did not affect the cell viability despite a higher electron density was observed in the cytoplasm. Similarly, the irradiation alone did not alter both viability and cell morphology (not shown). On the other hand, after irradiation in the presence of PS **4** (5 μM), morphological changes were observed at both envelope and cytoplasmic level. The outer membrane of approx. 90% of the treated cells appears fuzzier and lacks the pronounced margin of the envelope of control cells. Also the cytoplasmic compartment underwent changes: in approx. 60–70% of the cells we observed honeycomb-like structures (Fig. 5C) and in fewer cells (approx. 10%) distinguished filamentary structures located in the nucleoid region (Fig. 5D); cytoplasmic regions less electron dense than those of the control samples were evident in approx. 90% of the cells (Fig. 5E). These peculiar ultrastructural changes were observed only in PS treated and irradiated cells, but not in control samples; this rules out the hypothesis of possible artifacts.

E. faecalis cells show more evident intracytoplasmic alteration with respect to *E. coli* ones, in accordance with the higher

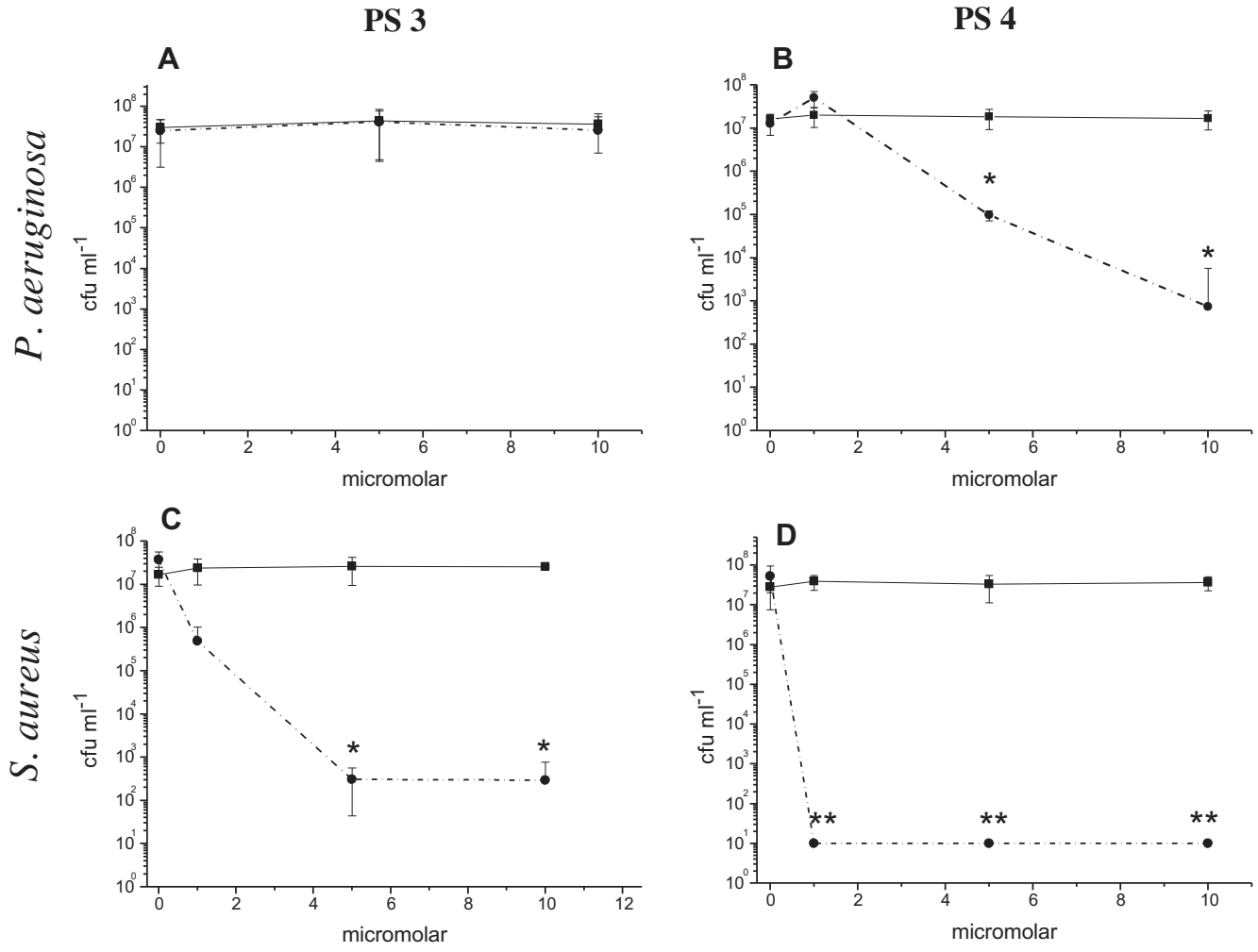


Fig. 3. Effect of increasing concentrations of PS **3** (A and C) and PS **4** (B and D) on the photodynamic inactivation of pure cultures of *P. aeruginosa* (A and B) and *S. aureus* (C and D). Cell viability was checked after 1 h dark incubation with the indicated PS (solid lines) or after 1 h of dark incubation followed by 75 min of irradiation with halogen lamp (dotted lines). The data are the mean of three independent experiments ± SD. *p* values were calculated by means of ANOVA test. * $p < 0.05$ and ** $p < 0.01$ for + PS + light vs – PS – light samples.

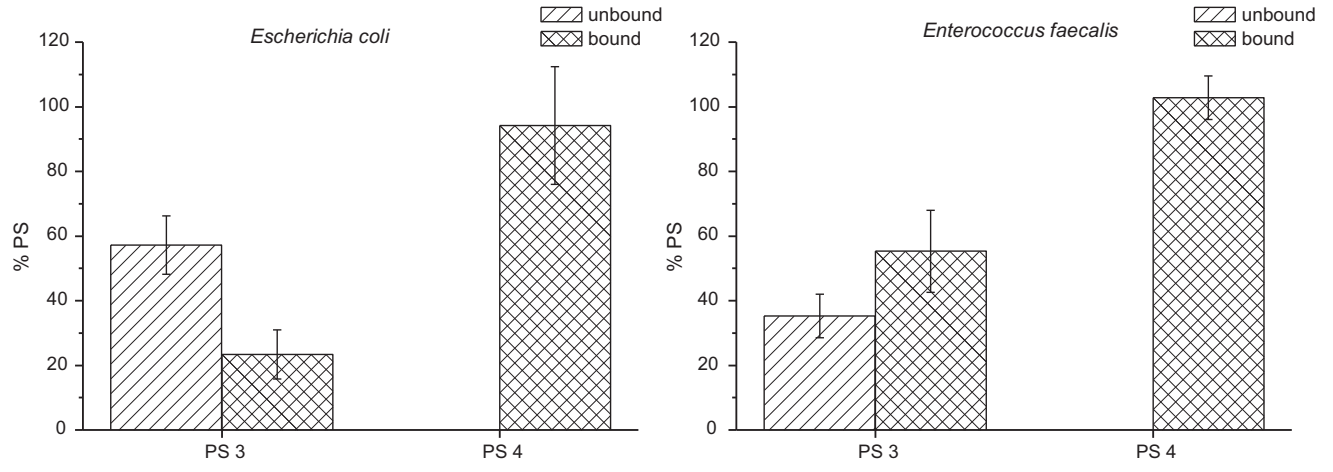


Fig. 4. Recovery of PS **3** or PS **4** from *E. coli* and *E. faecalis* cells after 1 h of dark incubation with the indicated PS (10 μM). The recovery from *E. faecalis* of all the administered amount of PS **4** was achieved using a two step extraction (see text for details). The percentages of bound and unbound PS are the mean of three independent experiments ± SD.

susceptibility of this microorganism to photoinactivation. Although 5 μM PS **4** was not intrinsically toxic to *E. faecalis* cells (Fig. 6B), micrograph images were fuzzy and cells looked different from those of the untreated and not irradiated control (Fig. 6A), exhibiting ultrastructural alterations such as a less dense cell wall and presence of low-density areas in the cytoplasm. Upon irradiation, in quite almost the fields analyzed, PS treated *E. faecalis* cells showed a filamentary organization of the nucleoid region (Fig. 6C)

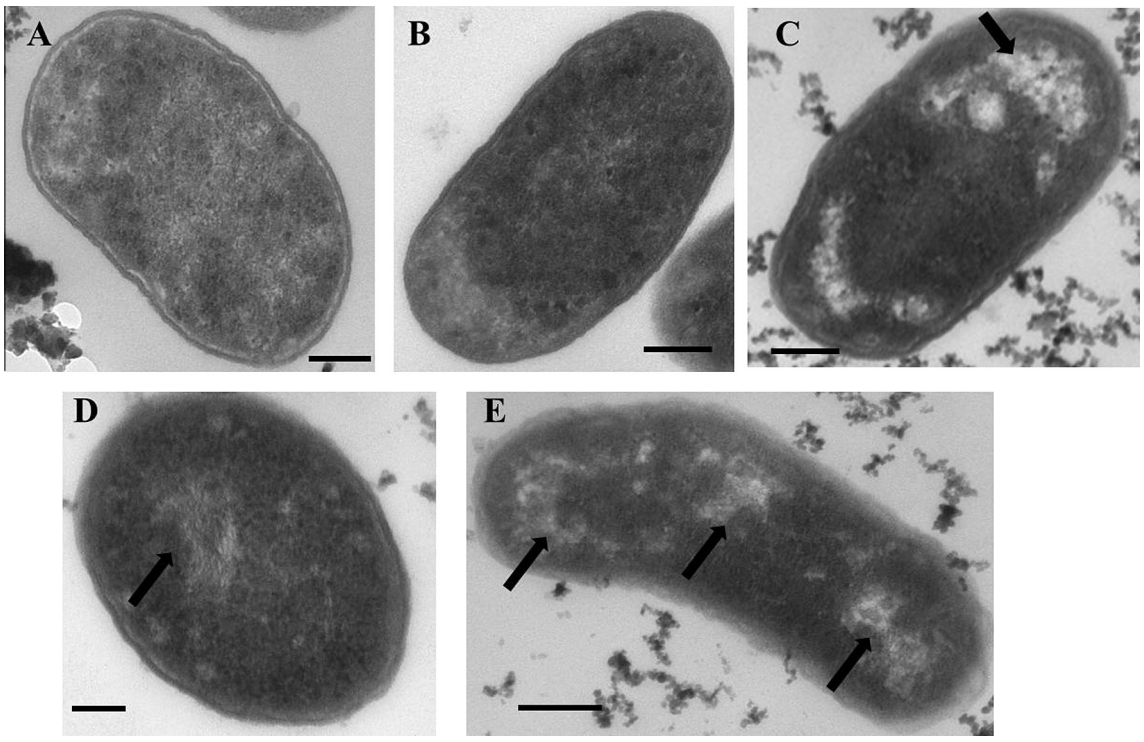


Fig. 5. Representative TEM micrographs of *E. coli* cells photosensitized for 10 min with 5 μM PS **4**. A, – PS – light; B, + PS – light; C, D, E, + PS + light (bar = 500 nm).

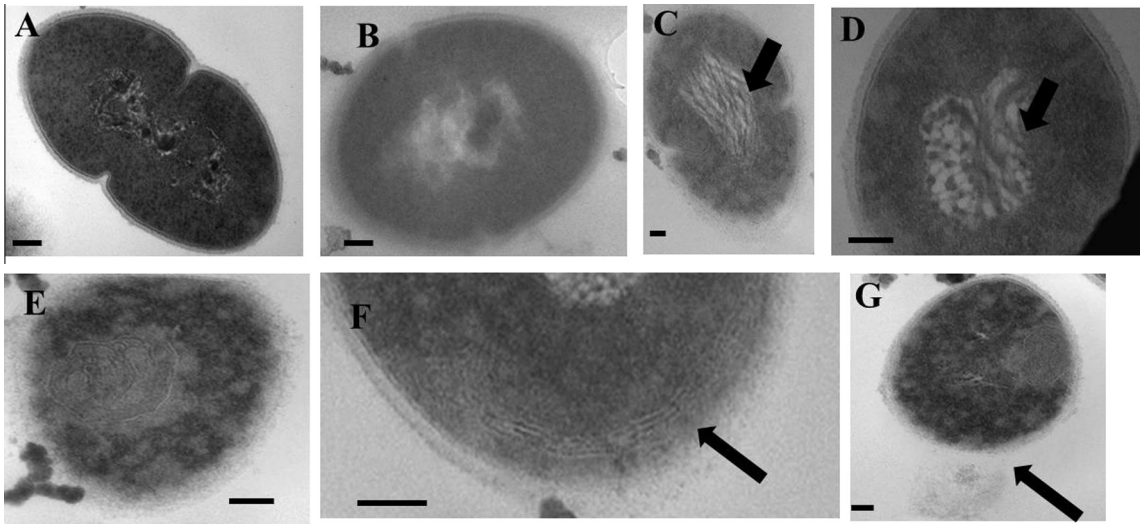


Fig. 6. Representative TEM micrographs of *E. faecalis* cells photosensitized for 10 min with 5 μM PS **4**. A, – PS – light; B, + PS – light; C, D, E, F, G, + PS + light (bar = 100 nm).

or, alternatively, a honeycomb-like structure (Fig. 6D and E) not present in the control samples (Fig. 6A). In photosensitized *E. faecalis* cells, a further peculiar structure was found consisting of folded structures parallel to the inner face of plasma membrane (Fig. 6F). The alterations of the cytoplasmic membrane could also be responsible for the formation of the bubbles or for the leakage of the inner content observed in only few *E. faecalis* cells (Fig. 6G), but not in *E. coli*.

3.5. Potential of PS 4 in the disinfection of bacterial water contaminants

For a deeper investigation on PS **4** potentialities, the efficiency of halogen and sun lights in photoinactivation experiments against mixed suspensions of *E. coli* and *E. faecalis*, bacteria regarded as indicators of faecal contamination, was compared (Fig. 7). Outdoor experiments were performed during the winter season (light intensity ranging from 28,000 to 53,000 lux and air temperature from 5 °C to 9 °C), administering PS **4** at 5 μM, the concentration which determined in the indoor experiment the greatest cell decrease detectable in the system (6 log units). To rule out any UV-A and UV-B bactericidal action, UV filter screened controls were also set up. As reported in Fig. 7, the viability of both *E. coli* and *E. faecalis* in mixed suspensions was neither affected by irradiation itself nor by 5 μM PS **4** in the absence of irradiation. In PS treated samples a 6 log unit viability reduction was achieved for both microorganisms regardless the light source used.

Similar experiments were also carried out on the effluents of an activated sludge treatment plant, collected after the secondary

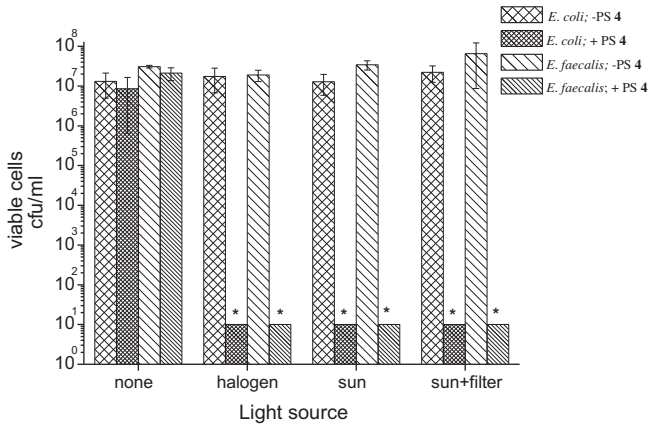


Fig. 7. Effect of photodynamic treatment with 5 μM PS **4** on mixed cultures of *E. coli* C1a and *E. faecalis* ATCC 51298. The viability of the two microorganisms was checked by plate count on specific selective media after 1 h incubation in the dark or after dark incubation followed by 75 min irradiation with either halogen lamp, sunlight, or UV-screened sunlight. The data are the mean of three independent experiments ± SD. *p* values were calculated by means of ANOVA test. * $p < 0.05$ for + PS + light samples versus - PS – light samples.

sedimentation, to check the effectiveness of photosensitization with PS **4** against wild microorganisms in a complex matrix. After 75 min of exposure, an average 2 log unit decrease of heterotrophic bacteria and of faecal coliforms was observed independently from the irradiation conditions. The treatment was more efficient against Enterococci as their population density decreased up to the detection limit (Table 1). Two fold increasing of the irradiation time (i.e. doubling the light dose) resulted, at best, in a further decrease of only 1 log unit of the monitored microflora (Table S1 and Fig. S2 in Supplementary data).

4. Discussion

The efficacy of porphyrin photosensitizers in photodynamic antimicrobial treatment depends on several structural and chemico-physical parameters. The presence of positive charges on the PS is mandatory to achieve a good activity against Gram negative bacteria [29], as positive charges promote a tight electrostatic interaction with negatively charged sites at the outer surface of the bacterial cells. The PS stuck on the bacterial cell surface exerts an efficient photodynamic action because the short living singlet oxygen, generated by energy transfer from the PS in the excited state to the molecular oxygen, easily reacts with the organic molecules of the cell wall thus impairing its integrity [13,30]. On the other hand, PS–cell interaction can be enhanced using molecules endowed with a certain degree of lipophilicity, that facilitates their penetration through cell membranes [32]. Hussain observed that among the cationic phenothiazinium-based compounds (PhBPs), the somewhat lipophilic ones ($\log P > 0.5$) were more active with respect to the hydrophilic congeners against the Gram-negative *E. coli* [7]. Among the previously synthesized tetracationic porphyrins already reported [33] the one featuring four benzyl groups as alkylating agent of the pyridyl nitrogen (5,10,15,20-Tetra(N-benzyl-4-pyridyl)porphyrin tetrachloride, TBzPyP) was found the most active, although its logP was negative ($\log P = -1$). In the same paper the activity of one dicationic congener serendipity isolated by partial alkylation of the tetrapyridyl porphyrin moiety was also described. This last compound was more efficient than the TBzPyP and its log*P*, although still negative, was the highest of all the tested cationic compounds ($\log P = -0.52$).

To study cationic PSs characterized by the presence of two positive charges only the structure of the 5,15-dipyridylporphyrin, from which the corresponding dicationic compounds can be easily and unequivocally achieved following N-alkylation, was conceived. The general synthetic pathway proposed by Boyle [26] was chosen for the synthesis of compound **2** as it requires the preparation of only one dipyrrolylmethane intermediate that can undergo cyclization to porphyrin *via* condensation with a single carbon atom reagent, such as formaldehyde or orthoformate. Concerning the low isolated yield of the porphyrin **2**, that one obtained (2%) is not too low with respect to that one reported in the literature (8%, [16]). Actually it is known that condensation yields of electron deficient aldehydes (such the 4-pyridinecarboxaldehyde) with pyrrole are by far lower than those obtained with electron rich aldehydes [16]. Indeed, in previously published syntheses of 5,15-diarylporphyrins, in which the aromatic moieties belonged to the nitro-substituted benzaldehydes, the yields of the isolated porphyrins were about 5% [34]. In analogy, it is not surprising that dipyrrolylmethane bearing the pyridine on "meso" position hardly reacts with orthoformate to afford the desired porphyrin. It must be pointed out that, in order to overcome this drawback, we also made a few attempts following a different synthetic approach (data not shown) in which the "meso" unsubstituted dipyrrolylmethane was made to react with the 4-pyridinecarboxaldehyde. Unfortunately, the yield of the isolated porphyrin **2** was comparable to that one of the above mentioned synthetic pathway.

The last step of the synthetic effort concerned the alkylation of the neutral porphyrin **2** with two different alkyl halides. This reaction has been successfully carried out in the presence of a large excess of alkyl halide used as solvent, thus the two dicationic

Table 1
Effect of photodynamic treatment with 5 μM PS **4** and 75 min irradiation on the microflora of three independent wastewater samples collected after the secondary sedimentation.

	−PS **4**			+PS **4**					
	Heterotrophic bacteria (PCA) (CFU/mL)	Faecal coliform (mFC agar) (CFU/mL)	Faecal enterococci (KF agar) (CFU/mL)	Heterotrophic bacteria (PCA) (CFU/mL)	Log unit reduction	Faecal coliform (mFC agar) (CFU/mL)	Log unit reduction	Faecal enterococci (KF agar) (CFU/mL)	Log unit reduction
60′ dark	1.8×10^5	6.7×10^4	8.0×10^3	1.0×10^5	0	4.4×10^4	0	2.6×10^3	0
	4.9×10^5	7.0×10^4	1.9×10^4	3.3×10^5	0	4.0×10^4	0	2.6×10^3	1
	4.6×10^5	5.5×10^4	1.5×10^4	4.4×10^5	0	8.0×10^4	0	3.3×10^3	1
75′ sun	8.3×10^5	1.5×10^4	1.3×10^3	1.8×10^4	1	30	3	<10	3
	1.5×10^5	5.0×10^3	3.2×10^3	6.0×10^3	2	8.0×10^2	1	<10	3
	1.5×10^5	2.5×10^4	4.1×10^3	1.5×10^3	2	1.8×10^2	2	<10	3
75′ sun + filter	1.2×10^5	4.6×10^4	3.2×10^3	1.5×10^4	1	50	3	<10	3
	1.7×10^5	1.4×10^4	4.6×10^3	2.5×10^3	2	7.0×10^2	2	20	3
	1.7×10^5	1.5×10^5	2.0×10^3	5.0×10^3	2	4.5×10^2	3	<10	3
75′ halogen lamp	6.7×10^4	2.8×10^4	4.5×10^3	4.0×10^4	0	4.0×10^2	2	<10	3
	2.2×10^5	3.0×10^4	2.8×10^3	1.7×10^3	2	4.0×10^2	2	<10	3
	3.0×10^5	1.1×10^5	5.0×10^3	6.0×10^3	2	3.2×10^3	2	<10	3

porphyrins, the methylated derivative PS **3** and the benzylated one PS **4** could be isolated by filtration following precipitation.

The chemico-physical studies of the two porphyrins indicate that the two compounds are characterized by similar photostability and singlet oxygen production rate but different lipophilicity. Both compounds showed a positive log*P* values and, in particular, compound **4** showed a log*P* > 0.5, the value indicated by Hussain et al. [7] necessary to obtain a good interaction with Gram negative bacteria.

PS **3** and PS **4** were first tested against pure cultures of Gram negative, *E. coli* and *P. aeruginosa*, and Gram positive, *E. faecalis* and *S. aureus*, bacteria. Independently from the PS used, the Gram negative bacteria were, as expected, less sensitive to the photodynamic treatment than the Gram positive ones. The lower sensitivity of Gram-negative bacteria is well documented and it has been ascribed to the structure of the cell wall and, in particular, to the presence of the outer membrane which makes these microorganisms less prone to PS penetration [5,6]. The presence of benzylic groups makes PS **4** more lipophilic than PS **3** thus more suitable to interact with the Gram negative lipopolysaccharidic outer membrane, and, ultimately, active also against these bacteria. It is also noticeable the absence of toxicity in the dark of both these new compounds up to 10 μM, whereas the TBzPyP previously reported showed a clear dark toxicity (unpublished data). At the same time the comparison of the photodynamic efficacy of the dicationic porphyrin **4** is higher with respect to that one of the TBzPyP determined under comparable experimental conditions.

Accordingly to what expected on the basis of PS lipophilicity, in *E. coli*, binding experiments showed that almost the whole amount of PS **4** administered was recovered after cellular lysis, whereas the PS **3** was remarkably less bound to the cells. Also in *E. faecalis* the entire amount of PS **4** was found bound to the cells, but only 50% could be recovered following a SDS treatment, the remaining fraction could be recovered only after the HCl 0.1 M treatment, suggesting that the PS is strongly bound to the cells. The PS **3**, more polar than PS **4**, showed a lower binding degree to the Gram-positive cells as 35% was recovered in the supernatant.

The comparable singlet oxygen production by the two PSs is not surprising as the peripheral substituents present on these porphyrins can hardly influence the electronic excitation and relaxation pathways of the tetrapyrrolic ring. As the consequence, the higher efficiency in photokilling of PS **4** with respect to PS **3** seems to be mainly ascribable to a better interaction with the bacterial cells.

At the early stages of the photoinactivation process, the cells treated with PS **4** underwent significant morphological alterations leading to cell death. The intracellular alterations detected were greater and more diffuse in the Gram positive *E. faecalis* cells than in *E. coli* cells, in agreement with the observations of Nitzan on the Gram positive model bacterium *S. aureus* and the Gram negative *E. coli* following photoinactivation with δ-ALA [34]. In *E. faecalis*, as in *S. aureus* [35], honeycomb like structure were observed. In *E. coli* the cell envelop appeared to be compromised and the cytoplasm appearance was quite different from that of healthy cells. Pudziuvyte et al. [36] observed similar alterations after irradiation of *E. coli* KMY1 treated with tetra-(4-ethylpyridynium)porphyrin tetratosylate; the authors assumed that this damage could increase the outer membrane permeability, thus favoring porphyrin cell penetration that in turn results in more photo-oxidative damages.

The mechanism of prokaryotic cell killing exerted by the photodynamic treatment makes this procedures a powerful mean to reduce bacterial cell number, which can be potentially applied to different matrices [37–38]. To these purposes the use of an economic and broad spectrum light source is advisable. We thus investigated whether the combination of sunlight with PS **4**, could be efficiently used in the photoinactivation of mixed cultures of the model organisms *E. coli* and *E. faecalis*.

In the outdoor experiments the sunlight of bright winter days was found as efficient as the halogen lamp in the photoinactivation of a mixture of the faecal indicators *E. coli* and *E. faecalis*; furthermore the UV contribution to this process appeared to be negligible as comparable results were obtained shielding the culture with an UV filter. When the same protocol was applied to wastewater samples, a good killing yield was obtained for all the monitored components of the microflora, although lower than that achieved with the mixed cultures of the model microorganisms *E. coli* and *E. faecalis*. Such lower efficiency is probably ascribable to the presence of particulate matter which can trap some of the PS, making it less available to the cells. The presence of organic matter has been demonstrated to negatively affect the binding of a tetracationic porphyrin to *P. aeruginosa* and thus its efficacy after irradiation [40]. The quite low reduction of the heterotrophic subpopulation can account for the presence of a high proportion of Gram negative bacteria such as strains related to Pseudomonads, that, in general, undergo photoeradication with great difficulty. On the contrary, the subpopulation of wild Enterococci showed a higher sensitivity than heterotrophic and faecal coliforms bacteria, to PS itself and to irradiation, as also reported by other authors [41,41]. PS **4** can thus be considered potentially useful in the disinfection of bacteria contaminated waters, and its potentiality is strengthened by the possibility to use solar light for the photoactivation. However, the feasibility of such application needs the study of new synthetic procedures aimed to increase the yield to reasonable values and a broader investigation of its antimicrobial activity.

The present study confirms that the new dicationic, 5,15-dipyridylporphyrins represent a class of photosensitizing agents endowed with an efficient antibacterial activity on different bacterial species under different irradiating conditions and at concentrations as low as the tetracationic congeners previously reported [33]. On the whole the results pointed out that a limited number of positive charges together with a certain degree of lipophilicity enhance the interaction of the PS **4** with the bacterial cells. This better interaction can be responsible for the higher efficacy of this PS against both Gram positive and Gram negative bacteria.

5. Abbreviations

PDT	photodynamic therapy
PS	photosensitizer
O/N	over/night
LB	Luria Bertani Broth

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jphotobiol.2013.08.011.

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