



Cationic photosensitizers and potassium iodide: an innovative approach for enhanced photodynamic inactivation of pathogenic bacteria in aquaculture

Cátia Vieira^a, Maria Bartolomeu^a, Carlos J.P. Monteiro^b, Jesús L. Romalde^c, Pedro P. Gallego^d, M. Graça P.M.S. Neves^b, M. Amparo F. Faustino^{b,*}, Adelaide Almeida^{a,*}

^a CESAM, Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

^b LAQV-REQUIMTE, Department of Chemistry, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal

^c Cross-disciplinary Research Center in Environmental Technologies (CRETUS), Department of Microbiology and Parasitology, CIBUS-Faculty of Biology, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain

^d Agrobiotech for Health, Plant Biology and Soil Science Department, Biology Faculty, University of Vigo, Vigo, Spain

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ABSTRACT

Aquaculture is a rapidly growing industry, accounting for more than half of the worldwide fish production. However, this sector is often affected by bacterial infections, threatening farmed animals and consumer safety. This study explored the potential of Photodynamic Inactivation (PDI) as an innovative antimicrobial approach for the inactivation of Gram-negative bacteria relevant to the aquaculture sector: *Vibrio parahaemolyticus*, *Vibrio anguillarum*, and *Escherichia coli*. The experiments were conducted in the presence of the cationic photosensitizers (PSs) 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (TMPyP) and methylene blue (MB). For *V. parahaemolyticus*, the protocol was extended to the neutral and anionic PSs curcumin (CUR), chlorin *e*₆ tri-methyl ester (Ce₆Me), and 5,10,15,20-tetrakis(4-sulfophenyl)porphyrin (TPPS₄). The assays were performed in the absence or presence of potassium iodide, well-known to improve PDI effect, while its potential for aquaculture application is yet to be studied. These evaluations were performed in phosphate-buffered saline (PBS) and in artificial seawater (ASW) to simulate aquaculture water disinfection. The cationic PSs TMPyP and MB were the most efficient in the bacterial inactivation. Although higher concentrations of PS were required to achieve effective bacterial inactivation in ASW compared to PBS, the application of KI enhanced the effectiveness of all PSs, reducing the treatment time at least by threefold. The bacterial inactivation profiles revealed higher susceptibility of *Vibrionaceae* bacteria which may be linked to the differential PDI impact on the outer cell membranes of these bacteria. These findings indicate that treatments with PSs, particularly TMPyP and MB, in combination with KI constitute a potential approach for bacteria control in the aquaculture sector.

1. Introduction

With the decline of wild fish stocks and the increase of the global population, aquaculture has emerged as one of the fastest-growing sectors, supplying nearly half of the world's seafood (FAO, 2022). However, this industry is often threatened by bacterial outbreaks that cause high mortality rates among farmed animals, resulting in severe economic losses (Hegde et al., 2023; Maldonado-Miranda et al., 2022). Vibriosis ranks among the most prevalent diseases in the aquaculture sector, often manifesting as a systemic infection characterized by

lethargy, loss of appetite, and skin ulcerations in affected animals (Manchanayake et al., 2023; Mohamad et al., 2019). It is caused by Gram-negative bacteria from the *Vibrionaceae* family, which are indigenous to aquatic environments and opportunist pathogenic for fish and shellfish (Manchanayake et al., 2023; Mohamad et al., 2019). Certain species within this family, such as *Vibrio parahaemolyticus*, *Vibrio cholera*, and *Vibrio vulnificus*, can also pose significant health risks to humans if raw or undercooked contaminated seafood is consumed (Brauge et al., 2024; Kim and Lee, 2017). Moreover, due to poor sanitation practices in fish farms and faecal contamination of aquatic environments,

* Corresponding authors.

E-mail addresses: catiavieira@ua.pt (C. Vieira), maria.bartolomeu@ua.pt (M. Bartolomeu), cmonteiro@ua.pt (C.J.P. Monteiro), jesus.romalde@usc.es (J.L. Romalde), pgallego@uvigo.es (P.P. Gallego), gneves@ua.pt (M.G.P.M.S. Neves), faustino@ua.pt (M.A.F. Faustino), aalmeida@ua.pt (A. Almeida).

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aquaculture can also serve as a source of *Enterobacteriaceae* bacteria and other non-indigenous fish microorganisms. (Immanuel Suresh et al., 2022; Onmaz et al., 2020) Although rarely infectious for animal stocks, they can be highly pathogenic to consumers if not effectively eliminated from fish farms (Elsherief et al., 2014; Mumbo et al., 2023). Therefore, the control of bacterial outbreaks in aquaculture production is essential for minimizing farmed fish mortality and ensuring consumer safety.

Disease control in aquaculture mainly relies on vaccination and antibiotics (Bedekar and Kole, 2022; Imtiaz et al., 2023; Irshath et al., 2023). Although vaccines are commonly utilized for fish immunization, they possess limitations including short-term immunity requiring re-administration and incomplete coverage against all fish pathogens (Bjørge et al., 2011; Chettri et al., 2015; Dang et al., 2021). On the other hand, antibiotics are commonly used for treating farmed fish, however, their application in aquaculture is highly discouraged due to their accumulation in aquatic environments, their potential to induce bacterial resistance, and to cause human health issues (Yuan et al., 2023; Zhang et al., 2021). Since aquaculture waters might serve as a primary reservoir of multidrug-resistant bacterial pathogens, recirculating aquaculture systems also utilize water disinfection treatments such as UV-C radiation (direct photolysis) and ozonation (O_3) to reduce the risk of infections among farmed fish (Rurangwa and Verdegem, 2015). However, these techniques also present major drawbacks, including the production of toxic by-products and the induction of genetic damage to several organisms (Jensen et al., 2011; Xin et al., 2023). To enhance the environmental and economic sustainability of aquaculture, there is an urgent need for the development of innovative treatment technologies.

Over the years, antimicrobial Photodynamic Inactivation (PDI) treatment has been gaining attention as a potential approach for environmental applications (Bartolomeu et al., 2021, 2017; Lopes et al., 2020), including aquaculture (Arrojado et al., 2011; Magaraggia et al., 2006; Malara et al., 2017a). PDI relies on the application of a photosensitizer (PS) that can be activated by visible light in the presence of dioxygen (3O_2), leading to the production of reactive oxygen species (H_2O_2 , $O_2^{\cdot-}$, OH^{\cdot} , 1O_2 , among others) (Cieplik et al., 2018). These oxidative chemical entities can damage the external structures of bacterial cells leading to their inactivation without promoting the development of resistant mechanisms (Alves et al., 2014a; Bartolomeu et al., 2016). Moreover, the efficacy of PDI treatments is not affected by the bacterial antibiotic resistance profile (Almeida et al., 2014; Bartolomeu et al., 2016).

To establish a successful PDI treatment for aquaculture applications, the selection of a PS is one of the most crucial factors to consider (Ghorbani et al., 2018). Previous *in vitro* studies have demonstrated that porphyrins (Alves et al., 2014b; Arrojado et al., 2011; Malara et al.,

2017a), phthalocyanines (Mantareva et al., 2022), xanthenes (Eshaghi Gorji and Li, 2022) and phenothiazinium dyes (Deng et al., 2016) exhibit remarkable antimicrobial activity against pathogenic bacteria of aquaculture systems. Recent research have also highlighted the successful utilization of natural PS, such as curcumin, riboflavin or polylactic acid/5-aminolevulinic acid in the decontamination of seafood products without affecting their quality, consequently, improving their shelf life (Chen et al., 2021, 2024; Li et al., 2021). These data suggest that PDI treatment may not only serve as a potential approach for decontaminating aquaculture water, but also for providing safe fish products for consumers through post-harvest decontamination methods.

Over the years, several studies have focused on improving the antimicrobial efficiency of PDI (Bartolomeu et al., 2021; Hamblin and Abrahamse, 2018). One of these approaches involves the application of the inorganic salt potassium iodide (KI), which has shown efficacy in improving the photodynamic activity of different PS against bacteria, fungi, and viruses (Vieira et al., 2019, 2018). The enhanced effect is caused by the reaction between KI and 1O_2 , which is generated by the PS (Fig. 1). This interaction yields peroxyiodide ($HOOI_2^-$), which subsequently decomposes to generate highly potent reactive iodine species, including free iodine/triiodide (I_2/I_3^-), diiodide radical ($I_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), all known for their antimicrobial properties (Huang et al., 2017; Vecchio et al., 2015; Vieira et al., 2018). Although the combined action of KI and PDI treatments has been extensively investigated in the clinical field (Kashef and Hamblin, 2022; Vecchio et al., 2015; Wen et al., 2017), its application on aquaculture systems is yet to be studied.

The main goal of this study was to explore the potential of five known PSs (Fig. 2), alone and in combination with KI, to assess their efficacy and determine the optimal conditions for PDI future approaches in the aquaculture industry. To this purpose, preliminary experiments were conducted to assess the efficacy of the PDI treatments against Gram-negative bacteria commonly found in aquaculture: *V. parahaemolyticus* and *Vibrio anguillarum*, bacteria highly pathogenic to farmed fish and shellfish, as well as *Escherichia coli*, a faecal pathogen often associated with foodborne illness from seafood consumption. These studies were performed first in phosphate-buffered saline solution (PBS) and then in artificial seawater (ASW) using the cationic PS 5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin (*TMPPyP*) and methylene blue (*MB*) due to their recognized efficiency to eradicate Gram-negative bacteria (Deng et al., 2016; Martins et al., 2018; Vieira et al., 2018). The protocol in ASW was then extended to the neutral and anionic PSs curcumin (*CUR*), chlorin *e*₆ methyl ester (*Ce₆Me*), and 5,10,15,20-tetrakis(4-sulfo-phenyl)porphyrin (*TPPS₄*), allowing to evaluate the impact of the PS charge on the PDI efficiency. These PSs were chosen due to their

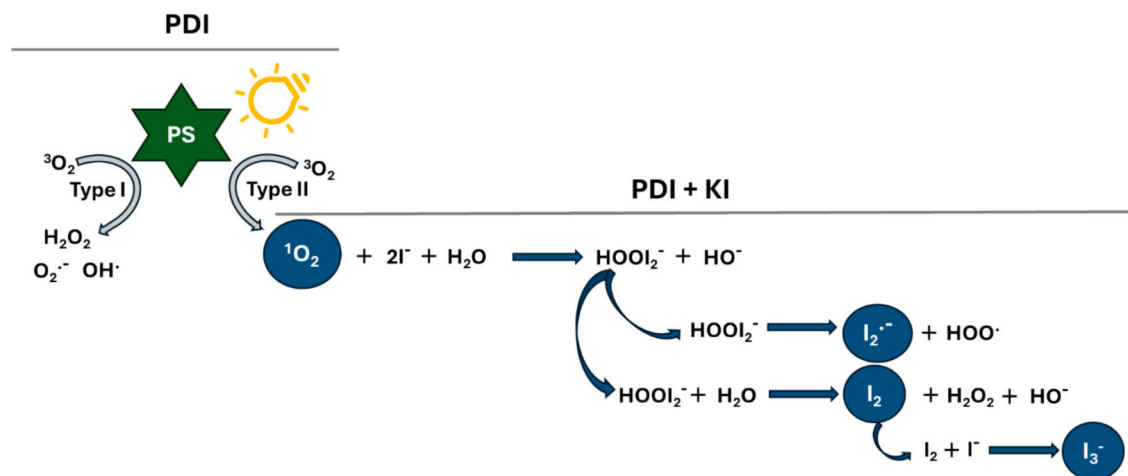


Fig. 1. Schematic representation of the PDI mechanism yielding 1O_2 and the production of reactive iodine species in the presence of KI. Adapted from Vieira et al. (2018).

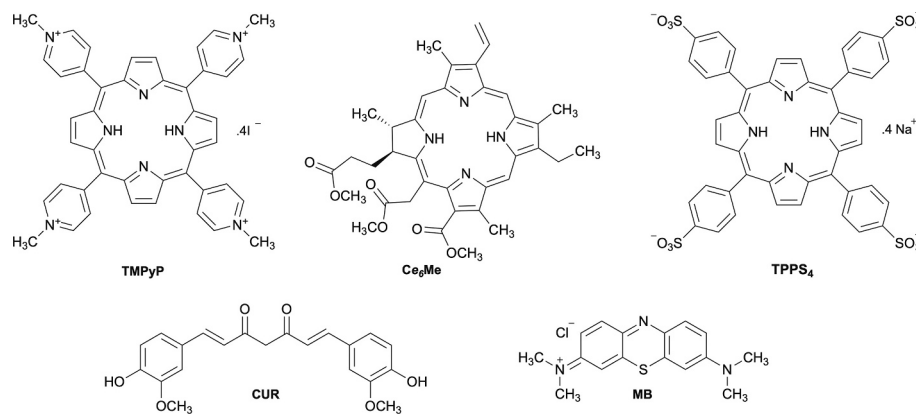


Fig. 2. Structures of the PS used in this study.

biocompatibility, low toxicity and extensively studied antimicrobial properties (Chen et al., 2022; Li et al., 2020; Mandys et al., 1998). *CUR* is a natural polyphenolic compound that can be extracted from *Curcuma longa* and was previously demonstrated to be efficient in the inactivation of microorganisms in food matrices (Li et al., 2020). On the other hand, the action of the compounds *Ce₆Me* and *TPPS₄* as efficient PS has been primarily focused on the clinical field (Chen et al., 2022; Mandys et al., 1998). For these assays was selected *V. parahaemolyticus* due to its high pathogenicity for both farmed fish and consumers (Ndraha et al., 2022), and the studies with each PS were also performed with and without KI.

2. Materials and methods

2.1. Photosensitizers and KI stock solution preparation

5,10,15,20-tetrakis(1-methylpyridinium-4-yl)porphyrin tetra-iodide (*TMPyP*), 5,10,15,20-tetrakis(4-sulfophenyl)porphyrin (*TPPS₄*) and chlorin e₆ methyl ester (*Ce₆Me*) were obtained according to the literature (Gonsalves et al., 1996; Monteiro et al., 2008; Simões et al., 2016; Uliana et al., 2014). *MB* and *CUR* were purchased from Acros Organics (Geel, Belgium). Stock solutions of each PS were prepared at 500 μM in dimethyl sulfoxide (DMSO) (*TMPyP*, *TPPS₄*, and *Ce₆Me*), sterile Milli-Q water (*MB*), and acetone (*CUR*), and kept in the dark. Before each experiment, the required solutions were sonicated for 30 min at room temperature (ultrasonic bath, SONOREX SUPER BK 102H, 35 kHz). Potassium iodide was purchased from Sigma-Aldrich (St. Louis, MO) and the stock solutions were prepared at 5 M in sterile Milli-Q water immediately before each assay.

2.2. Bacterial strains and culture conditions

The efficiency of photodynamic treatments was evaluated against the Gram-negative bacteria: *E. coli* ATCC 25922 strain obtained from the ATCC collection, *V. parahaemolyticus* and *V. anguillarum* strains previously isolated from Corte das Freiras farmed system in Ria de Aveiro (Pereira et al., 2011), Portugal (latitude 40°37'54.94"N & longitude 8°40'9.76"W), and from a turbot farm located in O Grove, Spain (latitude 42°28'16"N & longitude 8°51'42"W), respectively.

Bacterial strains were maintained at 4 °C on Tryptic Soy Agar (TSA, Merck) supplemented with 0 g/L NaCl for *E. coli*, 1 g/L of NaCl for *V. anguillarum* and 3 g/L of NaCl for *V. parahaemolyticus*. Before each assay, three isolated colonies were transferred to 30 mL of Tryptic Soy Broth (TSB, Merck) supplemented with the corresponding NaCl concentrations and incubated at 37 °C (for *E. coli* and *V. parahaemolyticus*) or 25 °C (for *V. anguillarum*) for 18 h under stirring (120 rpm). Then, 300 mL of the previous suspension was transferred to fresh TSB (30 mL) and incubated as previously described until the stationary phase of approximately 10⁹ colony forming units per mL (CFU.mL⁻¹) was

achieved. This culture was used for the biological assays.

2.3. Photodynamic Inactivation procedure

The photodynamic inactivation of the bacterial strains was evaluated with each PS alone at concentrations ranging from 0.1 μM to 5.0 μM, or in combination with KI at 100 mM. *CUR* and *Ce₆Me* were also tested at 20 μM due to their known instability under irradiation, which may require a higher concentration for a prolonged effect. The concentration of KI was selected as the most likely to improve PDI efficiency according to the literature (Vieira et al., 2018). The experiments were conducted on two suspension matrices: PBS and ASW (Tropic Marin®, see composition in Table S1).

Bacterial cultures of the stationary phase were 10-fold diluted in PBS or ASW. The microbial suspensions were then transferred to 12-well plates and an adequate volume of the PS was added to achieve the desirable concentration. For the assays performed in the presence of KI, this salt was also included. Simultaneously, different controls were also performed for each condition: light controls containing just the bacterial suspension (LC) or the bacteria plus KI (LC + KI) exposed to light; dark controls (DC) comprising a bacterial suspension incubated with each PS (at the highest concentration tested) and KI in the absence of light.

After samples and controls preparation, these suspensions were incubated in the dark for 15 min under stirring to promote the PS binding to the bacterial cells. Then, all samples were irradiated for 120 min with white light (380–700 nm, 100 mW.cm⁻² measured with a laser power meter Coherent® FieldMaxII-Top combined with a high-sensitivity thermopile power sensor Coherent® PS19Q) emitted by a LED system (LUMECO, 30 W, 2000 lm). The UV-Vis spectra of the PSs and LED emission spectrum are presented in Supplementary Fig. S1. In these assays, the dark control samples were shielded from light throughout the irradiation procedure.

Along the PDI treatments, aliquots of each sample and controls were collected at different time points (0, 5, 10, 15, 30, 45, 60, 90, and 120 min of irradiation). Afterwards, the aliquots were serially diluted in PBS or ASW and two droplets (10 μL each) per dilution were then plated on TSA supplemented with the appropriate NaCl concentrations. After 24 h of incubation at 37 °C, bacterial colonies were counted on the most appropriate dilution and the concentration of viable cells was expressed as CFU.mL⁻¹. When no viable colonies were present, it was considered that the bacteria were inactivated until the detection limit of the method (2 log CFU.mL⁻¹). This limit corresponds to the lowest concentration of bacteria detectable, accounting for the presence of a single colony per 10 μL plated. Three independent experiments were conducted with two replicates, and the results were averaged.

2.4. Evaluation of the amount of PS binding to the bacterial cells

Suspensions of *E. coli* and *V. parahaemolyticus* were prepared in PBS and incubated with *TMPPyP* and *MB* at 0.1 and 0.5 μM for 15 min in the dark. After incubation, bacterial cells were washed three times with PBS and centrifuged for 5 min at 13,000 g to remove the unbound PS. Then, the pellets were resuspended in a digestive solution containing 2% sodium dodecyl sulfate (SDS, Merck) and 0.1 M NaOH (Scharlau) and incubated at room temperature for 24 h, to promote the PS extraction of the cellular content and then the fluorescence was measured on a FluoroMax plus spectrofluorometer. For *TMPPyP*, the samples excitation was performed at 420 nm and their emission was collected at 655 nm, while for *MB* the samples were excited at 663 nm and the emission collected at 690 nm. The measured fluorescence intensity was then compared with a calibration plot built with known concentrations of each PS to determine the corresponding PS concentration bounded to the bacterial cells. Simultaneously, parallel aliquots of the suspensions incubated with the PS were centrifuged and washed as previously described. Then, for the determination of the concentration of viable cells, pellets were resuspended on PBS, serially diluted, drop plated on TSA, and incubated at 37 °C for 24 h. Experiments were conducted in triplicate for each condition and the results were expressed as the average number of PS molecules per CFU [nmol PS/Log(CFU)].

2.5. Outer membrane destabilization assays

The membrane stability of *E. coli* and *V. parahaemolyticus* under the PDI treatments was evaluated by using the SDS assay (Asok et al., 2012; Lok et al., 2006). To this purpose, bacterial cultures on the stationary phase were 10-fold diluted in PBS and distributed into 96-well plates. Then, appropriate volumes of *TMPPyP* or *MB* were added to achieve a final concentration of 0.5 μM and the absorbance was measured (time 0) at 600 nm using a Multiskan™ FC Microplate Photometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Controls containing only the bacterial suspension without PS were also performed. A SDS solution was added to reach a concentration of 0.1% and the plates were exposed to light using the same procedure described for the PDI experiments. The absorbance decay at 600 nm was measured along irradiation, and the percentage decrease in absorbance compared to the control cuvette reading was plotted against time. Three independent experiments were conducted with three replicates, and the results were averaged.

2.6. Dark and photostability

A fresh solution of each PS was prepared in AWS using quartz cuvettes and the absorbance was adjusted to ~ 1 at their respective λ_{max} values (420 nm and 436 nm, respectively). For the photodegradation assays, the PS solutions were exposed to the same irradiation conditions used in the biological assays (white light, 100 $\text{mW}\cdot\text{cm}^{-2}$) under magnetic stirring. The absorption spectrum was measured before (t_0 , Abs_0) and along irradiation (Abst) using a UV-Vis spectrophotometer (SHIMADZU UV-2501PC, Kyoto, Japan) within the range of 300–800 nm. Dark stability assays were conducted similarly, but the PS solutions were protected from light exposure. The results were expressed as the percentage of absorption decay, calculated as the ratio of absorbance at λ_{max} over different time periods compared to the initial absorbance before irradiation.

2.7. Statistical analysis

Statistical analysis was performed with the GraphPad Prism 7 software program. The normal distribution and homogeneity of variances of data were evaluated using the Kolmogorov-Smirnov and the Brown-Forsythe tests, respectively. To assess the significant differences between treated samples and their corresponding controls, data was treated with a 2-way ANOVA and Tukey's (PDI and outer membrane

stability experiments) or Sidak's (PS binding assays) multiple comparisons tests. Tests were performed at $\alpha = 0.05$ significance level, thus differences corresponding to $p < 0.05$ were considered significant. At least three independent assays were performed for each condition in duplicate.

3. Results

3.1. Photodynamic Inactivation assays in phosphate-buffered saline (PBS) solution

The results obtained from photoinactivation of *E. coli*, *V. anguillarum* and *V. parahaemolyticus* in PBS and in the presence of the well-established PS, *TMPPyP* and *MB* are summarized in Fig. 3. As already mentioned, these PS were considered in these initial assays due to their recognized efficiency in bacterial photoinactivation processes. The experiments were performed under white light irradiation (380–700 nm) for 120 min, in the presence of KI at 100 mM, or in its absence.

For all the experiments, no significant variation was observed in light controls (LC) and dark controls (DC) ($p > 0.05$) indicating that bacteria viability was not affected by light irradiation alone, by the presence of KI under irradiation, or by the PS at the highest tested concentration (5.0 μM) in combination with KI in the absence of light.

The results presented in Fig. 3 indicate that *TMPPyP* and *MB* were efficient at reducing the number of viable cells of *E. coli*, *V. anguillarum* and *V. parahaemolyticus*. At a concentration of 5.0 μM , PDI treatments with both PSs resulted in the photoinactivation of all the bacterial strains to the detection limit of the method (ca. 8 log CFU. mL^{-1} reduction, $p < 0.05$) after 5 min of irradiation. However, at the lower tested concentrations (0.5 and 0.1 μM), the pattern of photoinactivation varied among the bacterial strains.

Overall, *E. coli* exhibited lower susceptibility to PDI treatments compared to the other bacteria, requiring longer irradiation times. Treatments with *TMPPyP* and *MB* at a concentration of 0.5 μM resulted in the *E. coli* photoinactivation to the detection limit of the method (ca. 8 log CFU. mL^{-1} reduction, $p < 0.05$) after 30 and 45 min of irradiation, respectively. However, the combination of both PS (0.5 μM) with KI (100 mM) improved their efficiency ($p < 0.05$) allowing to reduce the treatment time to 5 min (Fig. 3A,B).

For *V. anguillarum* (Fig. 3C,D), PDI treatments with *TMPPyP* and *MB* at concentrations of 0.5 μM , caused a sharp decrease in *V. anguillarum* concentration in the first 10 min of irradiation (> 4.0 log CFU. mL^{-1} reduction), which reached the detection limit of the method 5 and 20 min later, respectively ($p < 0.05$). To determine the lowest concentration required to photoinactivate *V. anguillarum*, the PS concentration was reduced to 0.1 μM . However, at this concentration, only *TMPPyP* achieved a reduction in bacterial concentration superior to 3.0 log CFU. mL^{-1} ($p < 0.05$) after 120 min of irradiation. The combination of both PSs (*TMPPyP* and *MB*) with KI resulted in an enhanced photoinactivation effect against *V. anguillarum*. This combined effect was particularly notable at the lower tested PS concentration (0.1 μM), as the photoinactivation pattern observed resembled that obtained with PS alone at a concentration five times higher (0.5 μM).

V. parahaemolyticus showed the highest susceptibility to PDI treatments compared to other bacteria under study (Fig. 3E,F). Both PS, at concentrations 0.5 and 0.1 μM required shorter PDI treatment times to reduce the bacterial concentration to the detection limit of the method (ca. 8 log CFU. mL^{-1} reduction, $p < 0.05$). With *TMPPyP* at concentrations of 0.5 and 0.1 μM , the bacterium inactivation to the detection limit of the method was achieved after 10 and 45 min of PDI treatment, respectively. *MB* showed a similar trend, however, at a concentration of 0.5 μM , the reduction of *V. parahaemolyticus* was observed 5 min earlier. Similarly, to *V. anguillarum*, the action of KI proved to be advantageous when combined with both PSs at a concentration of 0.1 μM , reducing the treatment time by ca. threefold (from 45 to 15 min).

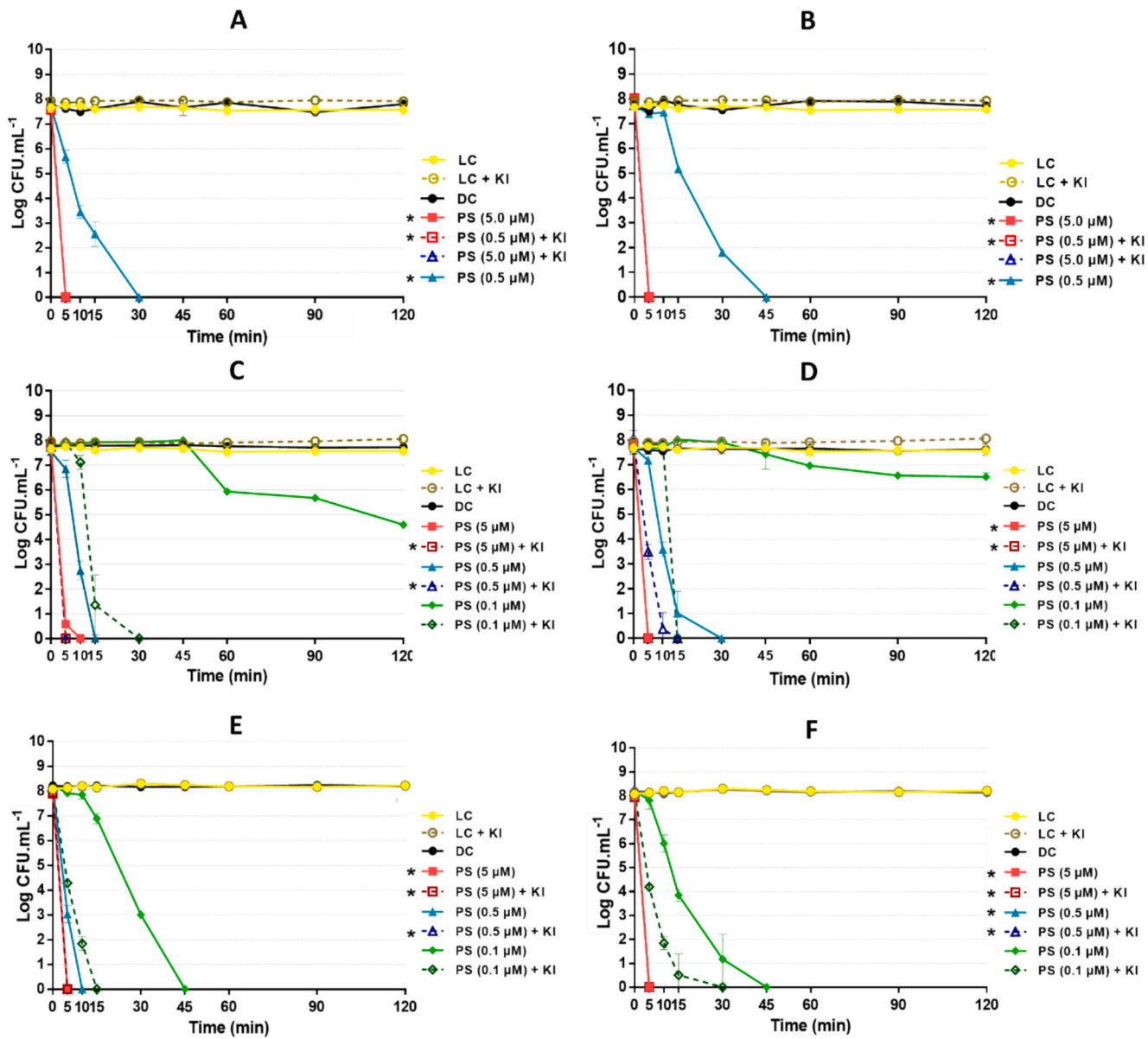


Fig. 3. Photodynamic inactivation of *E. coli* (A and B), *V. anguillarum* (C and D), *V. parahaemolyticus* (E and F) in PBS, using *TMPyP* and *MB*, respectively, alone and in combination with KI at 100 mM, under 120 min of white light irradiation at an irradiance of 100 mW.cm⁻². Each value represents the mean \pm standard deviation of three independent experiments with two replicates each (some of the error bars are hidden under the symbols). Treated samples exhibiting the same inactivation kinetics have overlaid lines and are represented with (*).

3.2. Photodynamic Inactivation assays in artificial seawater (ASW)

The effect of PDI treatments with *TMPyP* and *MB* on the photoinactivation of *E. coli*, *V. anguillarum*, and *V. parahaemolyticus* in AWS was assessed under the same irradiation conditions to those used in experiments conducted in PBS (Fig. 4).

The results shown in Fig. 4 reveal that the treatments in ASW required higher concentrations of both PSs to achieve an efficient photoinactivation of the tested bacteria, compared to PBS ($p < 0.05$). Both PSs, at 5.0 μM concentration, required 45 min of light irradiation to reduce the bacterial concentration of *E. coli* to the detection limit of the method (ca. 8 log CFU.mL⁻¹ reduction, $p < 0.05$) (Fig. 4A,B) while to reduce the bacterial concentration of *V. parahaemolyticus* it was required 30 min ($p < 0.05$) (Fig. 4E,F). Under the same light conditions, a similar reduction profile of *V. anguillarum* was observed after 15 and 30 min of PDI treatment with *TMPyP* and *MB*, respectively (Fig. 4C,D). In PBS, the

same photodynamic effect was observed with PS concentrations ten times lower (0.5 μM) than the used in ASW ($p < 0.05$); these results highlight the increased challenge of bacterial inactivation within this more complex matrix. At a concentration of 0.5 μM *TMPyP* and *MB* also demonstrated promising photodynamic activity leading to reductions in bacterial concentration exceeding 3.0 log CFU.mL⁻¹. However, *TMPyP* efficacy was particularly noteworthy against *V. anguillarum* and *V. parahaemolyticus*, causing the photoinactivation to the detection limit of the method (ca. 8 log CFU.mL⁻¹ reduction, $p < 0.05$) after 45 min of PDI treatment ($p < 0.05$) (Fig. 4C,E). These results not only highlight the superior effectiveness of *TMPyP* compared to *MB* but also the greater susceptibility of *Vibrionaceae* species compared to *E. coli*.

The combined action of KI also improved the photodynamic activity of both PSs against all the bacteria in AWS. In all cases, the photoinactivation achieved with the PS combination at 0.5 μM with KI surpassed that attained with the PS alone at 5.0 μM ($p < 0.05$). Therefore,

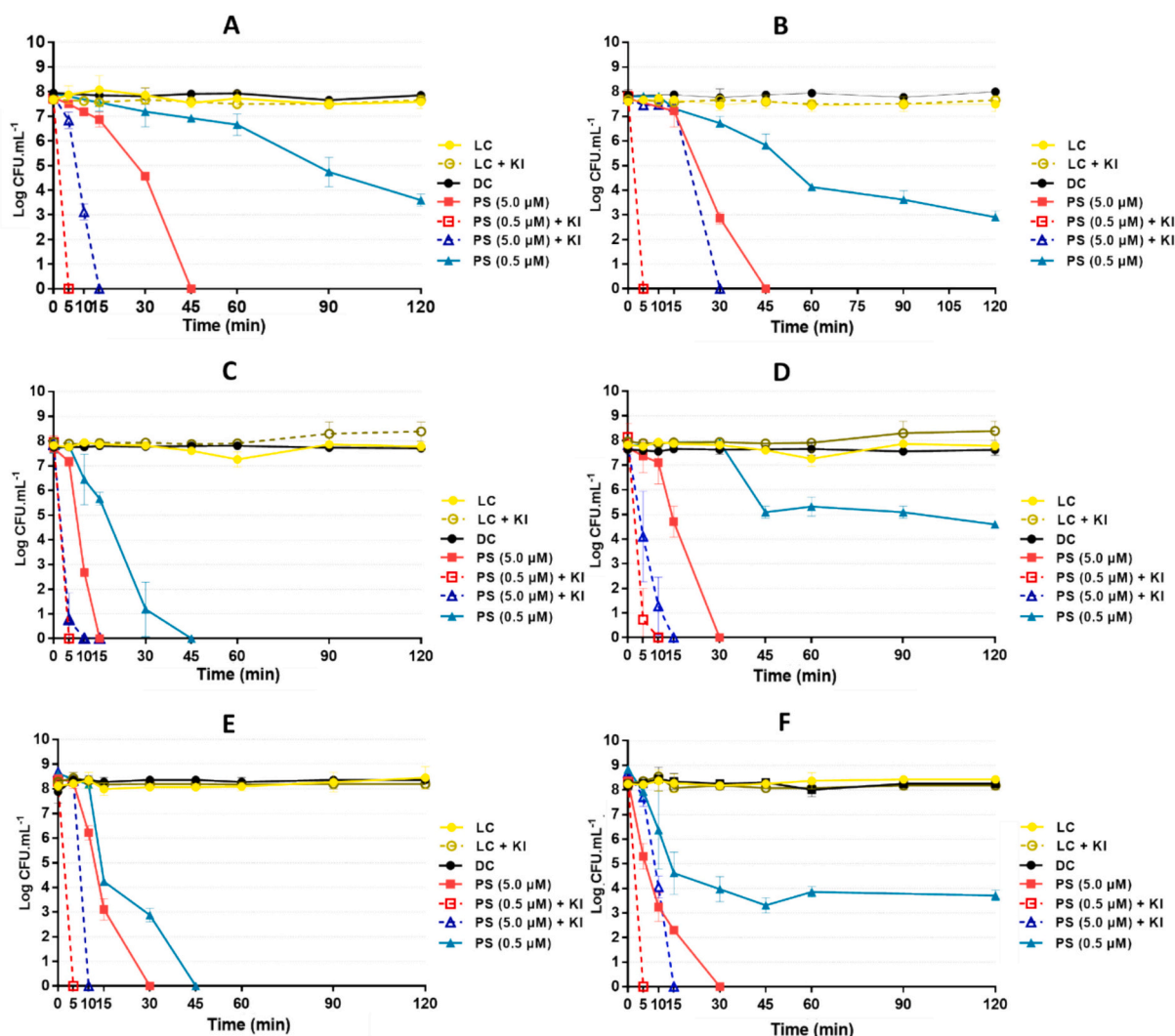


Fig. 4. Photodynamic inactivation of *E. coli* (A and B), *V. anguillarum* (C and D), *V. parahaemolyticus* (E and F) in ASW, using *TMPPyP* and *MB*, respectively, alone and in combination with KI at 100 mM, under 120 min of white light irradiation at an irradiance of 100 mW.cm⁻². Each value represents the mean \pm standard deviation of three independent experiments with two replicates each (some of the error bars are hidden under the symbols).

the presence of KI enabled a reduction in PS concentration by at least tenfold, indicating its potential as a valuable asset for PDI application in aquaculture.

Considering that *CUR*, *Ce₆Me*, and *TPPS₄* are also well-established PSs in the context of photodynamic-based applications, we decided to investigate how the photodynamic efficiency would be affected by the PS charge (neutral and negative charge) under conditions that reflect a realistic aquaculture scenario. In line with this, the efficacy of these three PS against *V. parahaemolyticus* was tested in ASW (Fig. 5). This pathogenic bacterium was selected among the others studied due to its negative impact on farmed stocks and consumers' health, as previously mentioned (Ndraha et al., 2022).

The photoinactivation profiles of *V. parahaemolyticus* show that *CUR*, *Ce₆Me*, and *TPPS₄* at 5.0 μM are less efficient than *TMPPyP* and *MB* at the same concentration ($p < 0.05$), but their efficiency can be improved by the presence of KI or in some cases by increasing the concentration to 20 μM (Fig. 5).

CUR was able to cause a 3.0 log CFU.mL⁻¹ reduction in *V. parahaemolyticus* concentration within the first 15 min of treatment at the two tested concentrations (20 and 5.0 μM) ($p < 0.05$), with no further inactivation improvement observed during the remaining treatment time ($p > 0.05$) (Fig. 5A). The PDI treatment with *Ce₆Me* at a concentration of 5.0 μM, showed no impact on *V. parahaemolyticus*

viability ($p > 0.05$), while at 20 μM, it achieved a 3.9 log reduction after 60 min of white light irradiation ($p < 0.05$) (Fig. 5B). Finally, *TPPS₄* achieved a maximum photoinactivation of 2.6 log ($p < 0.05$) after 120 min of white light irradiation at a concentration of 5.0 μM (Fig. 5C).

Overall, the combination of the *CUR*, *Ce₆Me*, and *TPPS₄* with KI enhanced their photodynamic activity against *V. parahaemolyticus*. At a concentration of 20.0 μM, *CUR* caused a 3.0 log CFU.mL⁻¹ reduction in bacterial concentration after 15 min of treatment. When combined with KI, the inactivation rate increased more than twofold, achieving a reduction of 6.7 log CFU.mL⁻¹. Similarly, *Ce₆Me* at the concentration of 20.0 μM, which alone failed to cause bacterial photoinactivation, achieved reductions to the detection limit of the method when combined with KI ($p < 0.05$). However, at the lowest tested concentration of both PS (*CUR* and *Ce₆Me* at 5.0 μM), no additional enhancement of photoinactivation activity was observed in the presence of this co-adjuvant ($p > 0.05$).

For the anionic *TPPS₄*, the use of KI resulted in bacterial photoinactivation to the detection limit of the method (ca. 8 log CFU.mL⁻¹ reduction, $p < 0.05$) after 5 and 60 min at PS concentrations of 5.0 and 0.5 μM ($p < 0.05$), respectively (Fig. 5C). Although the timeframe at 0.5 μM is longer compared to the 10 and 15 min achieved by *TMPPyP* and *MB* when combined with KI at the same PS concentration (Fig. 4E,F), the results obtained are particularly relevant considering the anionic

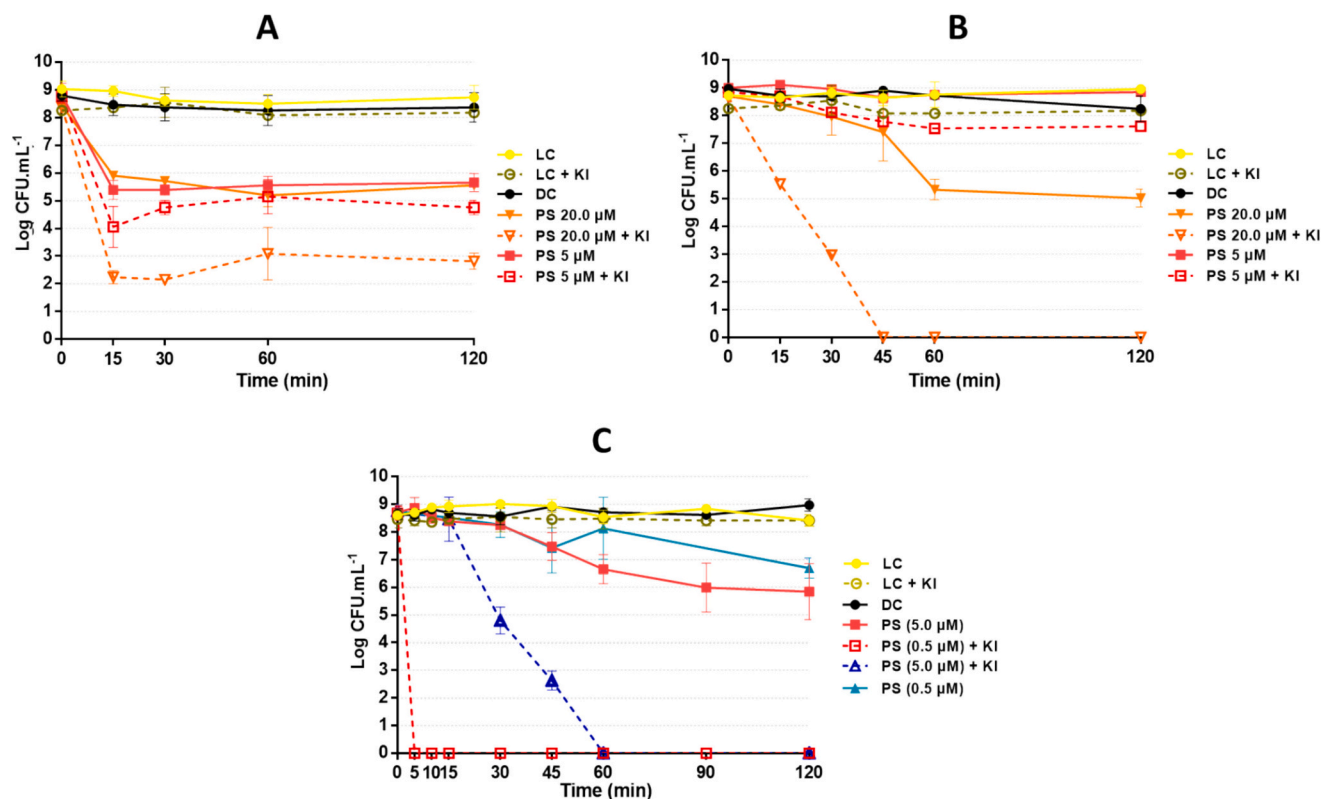


Fig. 5. Photodynamic inactivation of *V. parahaemolyticus* using CUR (A), Ce_6Me (B), $TPPS_4$ (C) in ASW, alone and in combination with KI at 100 mM, under 120 min of white light irradiation at an irradiance of $100 \text{ mW}\cdot\text{cm}^{-2}$. Each value represents the mean \pm standard deviation of three independent experiments with two replicates each (some of the error bars are hidden under the symbols).

features of the PSs. It is worth noting that $TPPS_4$ at 5.0 and 0.5 μM in the absence of KI, did not achieve bacterial inactivation to the detection limit of the method. However, this behavior changed with the presence of the co-adjutant salt.

LC and DC conducted throughout all PDI experiments in ASW remained stable ($p > 0.05$), indicating that bacterial concentration was unaffected by white light irradiation alone, the presence of KI, or the presence of the tested PS in combination with KI, at the highest tested concentration (5.0 μM), in the dark.

3.3. Evaluation of the factors influencing variability in bacterial susceptibility to PDI treatments

The previous results unveiled distinct susceptibilities of the studied bacterial strains to PDI treatments. The effective photoinactivation of *E. coli* required higher PS concentrations and longer PDI treatment, when compared with the conditions used for the inactivation of *V. anguillarum* and *V. parahaemolyticus*. In order to understand these distinct susceptibilities, we decided to evaluate if this is related to the preferential affinity of the PS for one bacterium over another or from differences in their cell wall sensitivity to the PDI treatments. In line with this, we investigated the binding features of each PS to bacterial cells and the effect of the PDI treatments on bacterial outer membrane destabilization. For these assays, we selected *E. coli* and *V. parahaemolyticus*, the bacteria with the most distinct susceptibility patterns among the three studied strains.

3.3.1. PS binding

To assess if the distinct susceptibility of *E. coli* or *V. parahaemolyticus* to PDI is related to the PS affinity to the cell walls, the amount of $TMPyP$ and MB attached to the bacterial cells was determined according to the protocol described in Section 2.5. The results obtained after incubating

E. coli and *V. parahaemolyticus* with $TMPyP$ and MB at concentrations of 0.5 and 0.1 μM for 15 min of dark incubation are shown in Fig. 6. In these assays, the PS concentration of 5.0 μM was not considered since both bacterial strains showed the same PDI outcome at this higher tested concentration.

This binding study revealed that the amount of $TMPyP$ and MB bound to *V. parahaemolyticus* cells was superior to that of *E. coli*. However, these differences were not significant ($p > 0.05$) (Fig. 6), suggesting that the PS affinity to the studied bacteria is not the only responsible factor for the different photoinactivation patterns observed.

The binding assays can also be a useful tool for comparing the binding values between the PS. The results obtained revealed that, overall, $TMPyP$ (0.5 and 0.1 μM) presented the highest cell binding values for both *E. coli* [2.4 and 2.1 nmol PS/Log (CFU)] and *V. parahaemolyticus* [4.1 and 3.1 nmol PS/Log (CFU)] cells. At the same concentrations (0.5 and 0.1 μM), MB cell binding on both bacteria was lower, with binding values of 2.0 and 1.2 nmol PS/Log (CFU) for *E. coli* and 2.6 and 1.9 nmol PS/Log (CFU) for *V. parahaemolyticus*, respectively.

3.3.2. Effect of PDI treatments on bacterial outer membrane integrity

In order to evaluate if the PDI treatment has a distinct impact on the destabilization of the *E. coli* and *V. parahaemolyticus* outer membrane, the susceptibility of both cell types to detergent-mediated cell lysis was analyzed before and after PDI treatment in the presence of $TMPyP$ and MB . If the outer membrane is compromised, treatment with sodium dodecyl sulfate solution (2% SDS) results in a sharp decline in optical density (OD_{600}) due to leakage of bacterial cell contents (Lok et al., 2006). On the other hand, in the absence of membrane damage, optical density remains stable.

The results obtained from the outer membrane destabilization assays are represented in Fig. 7 and indicate that PDI treatments led to a faster

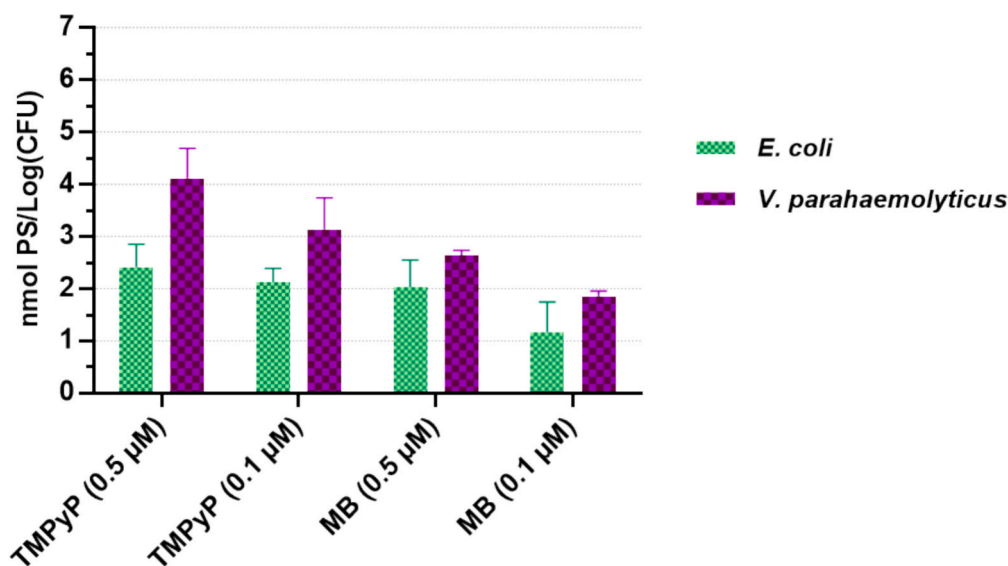


Fig. 6. Amount of *TMPyP* and *MB* binding to *E. coli* and *V. parahaemolyticus* cells after 15 min of dark incubation at concentrations of 0.5 and 0.1 μM . Values represent the mean of three independent assays in triplicate; error bars represent the standard deviation.

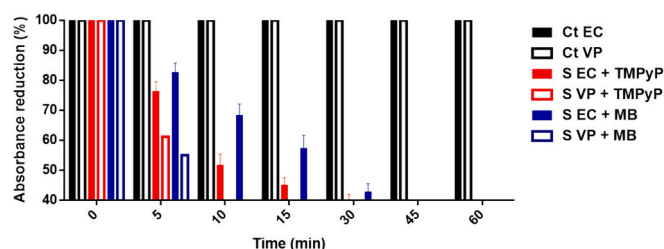


Fig. 7. Effect of PDI treatments with *TMPyP* and *MB* on *E. coli* (Ec) and *V. parahaemolyticus* (Vp) outer membrane destabilization along 60 min of white light irradiation at an irradiance of $100 \text{ mW}\cdot\text{cm}^{-2}$. Ct and S correspond to irradiated controls and treated samples, respectively. Values represent the mean of three independent assays in triplicate; error bars represent the standard deviation.

destabilization of the *V. parahaemolyticus* outer membrane compared to *E. coli*, resulting in a 60% decrease in *V. parahaemolyticus* integrity within 10 min of white light irradiation, for both PSs (*TMPyP* and *MB*) at a concentration of $0.5 \mu\text{M}$. In contrast, for *E. coli*, a similar effect was observed only after 30 min in the presence of *TMPyP* and after 45 min for *MB*.

4. Discussion

Aquaculture is a rapidly growing industry, accounting for more than half of the worldwide fish production (FAO, 2022). However, the high concentration of nutrients and dense animal populations within aquaculture facilities can promote the proliferation of pathogenic bacteria (Hegde et al., 2023; Maldonado-Miranda et al., 2022). This poses a risk not only to the health of farmed fish, but also to consumers who may be exposed to these pathogens through consumption (Dumen et al., 2020; Sheng and Wang, 2021). Previous studies have shown that PDI might be a possible solution to overcome this challenge, offering a promising approach for controlling infections in aquaculture facilities (Arrojado et al., 2011; Asok et al., 2012; Eshaghi Gorji and Li, 2022; Magaraggia et al., 2006; Notaro et al., 2021). Furthermore, this approach has also demonstrated promising results in a post-harvest stage for decontaminating seafood products (Chen et al., 2021; Eshaghi Gorji and Li, 2022; Gao et al., 2019; Li et al., 2021). To ensure the success of PDI in the aquaculture field, it is necessary to develop protocols capable of

achieving high bacterial inactivation rates with short treatments. To this purpose, this study explored the potential of PDI for the photoinactivation of bacterial strains relevant to aquaculture systems by using a wide range of PS at different concentrations and light doses. The effect of the photodynamic treatments was assessed with the PS alone and in combination with KI, a well-known co-adjuvant of PDI, which potential for aquaculture applications was herein evaluated for the first time.

Firstly, the activity of two extensively studied PSs *TMPyP* and *MB*, on the photoinactivation of *V. parahaemolyticus*, *V. anguillarum*, and *E. coli* was evaluated in a buffer solution, PBS, to confirm the antimicrobial activity of both PSs towards the selected bacterial strains. Then, the same PDI treatments were replicated in ASW mimicking aquaculture water composition. In these simulating real aquaculture conditions, our findings revealed that both PSs (*TMPyP* and *MB*) have high potential for the photoinactivation of the selected Gram-negative bacteria. However, higher concentrations of PS and/or longer treatments were required to achieve the bacterial inactivation to the detection limit of the method in ASW compared to PBS (Figs. 4 and 3, respectively). The lower efficiency of PDI treatments in seawater matrices was already reported in our research group (Bartolomeu et al., 2023). In 2023, Bartolomeu et al. have shown that *E. coli* reduction to the detection limit of the method (ca. $8 \log \text{CFU}\cdot\text{mL}^{-1}$ reduction, $p < 0.05$) was achieved sooner in PBS (30–45 min) than in real seawater and aquaculture water (60–120 min) when using a cationic porphyrinic formulation (Bartolomeu et al., 2023). This effect was associated with the higher concentration of dissolved salts in these lasts. Previous studies have shown that certain salts can contain specific ions capable of scavenging ROS and protecting against oxidative stress (Culotta and Daly, 2013; Yang and Pignatello, 2017). These ions can react with ROS produced during the PDI treatment through various mechanisms, including electron transfer reactions, radical-radical reactions, and redox reactions ultimately reducing the levels of ROS and diminishing bacterial oxidative damage. Indeed, the ASW used is composed by essential elements and trace elements from marine waters in natural proportions that may act as scavengers of ROS produced upon white light irradiation of *TMPyP* and *MB* under air conditions. Still, these PSs proved to be effective against aquaculture pathogens in seawater (Fig. 4), achieving bacterial inactivation to the detection limit of the method within 45 min or less of white light irradiation at concentrations as low as $5.0 \mu\text{M}$.

The comparison of photodynamic bacterial inactivation profiles revealed distinct susceptibility patterns among the studied strains. Overall, *V. parahaemolyticus* exhibited the shortest required treatment

time, followed by *V. anguillarum* and then *E. coli*. This higher susceptibility of *V. parahaemolyticus* has been observed in previous studies (Arrojado et al., 2011; Ma et al., 2020; Rafeeq et al., 2020; Wang et al., 2022). In 2011, Arrojado et al. showed that a tricationic porphyrin (Tri-Py⁺-MePF, 5.0 μM) effectively reduced *V. parahaemolyticus* concentration by approximately 6 log CFU.mL⁻¹ after 7.2 J.cm⁻² of white light exposure. In contrast, achieving a similar reduction for *V. anguillarum* and *E. coli* required twice the treatment duration (Arrojado et al., 2011). Another study also highlighted *V. parahaemolyticus*' higher susceptibility to photodynamic treatment with CUR compared to *E. coli*, *Listeria monocytogenes*, and *Shewanella putrefaciens* (Wang et al., 2022). In that case, a 4-times lower concentration of CUR (50 μM) was sufficient for effective photoinactivation of *V. parahaemolyticus* compared to the other species (200 μM).

To investigate the differences in bacteria susceptibility to PDI in this study, the PS affinity for bacterial cells and destabilization of the outer membrane during treatments were analyzed (Figs. 6 and 7). Our findings suggest that the different susceptibilities of *E. coli* and *V. parahaemolyticus* are primarily attributed to the differential destabilization of their bacterial membranes (Fig. 7) rather than PS binding (Fig. 6). The reason behind the disparity in bacterial membrane behavior during the PDI process may be attributed to differences in the composition of the microbial membranes of these strains. According to the literature, the ratio of unsaturated to saturated fatty acids in bacterial membranes influences the efficiency of PDI. Unsaturated fatty acids have been reported to be more prone to damage by ROS, the primary mechanism behind the photodynamic inactivation of bacteria, compared to saturated fatty acids (Kumar et al., 2016). Lambert and his co-workers observed differences in the fatty acid composition between *E. coli* and *Vibrionaceae* species (Lambert et al., 1983). The ratio of unsaturated to saturated fatty acids (16:1/16:0) in *Vibrionaceae* ranged between 1.5 and 3.9, while in *E. coli* this ratio was lower than 1. Therefore, it is possible that a higher content of unsaturated acid in the *Vibrionaceae* species studied may facilitate membrane oxidation and, consequently, lead to a faster inactivation. Previous studies conducted by Alves et al. using infrared spectroscopy monitored the changes produced by PDI treatment on *E. coli* by TMPyP (Alves et al., 2016). This study revealed that most of the changes occurred in proteins and lipids, suggesting a significant effect on lipopolysaccharides in the initial irradiation times. Similar results were obtained by the same group when they used lipidomic approach to identify the most significant damage found in *E. coli* structures after PDI with a tricationic porphyrin (Tri-Py⁺-Me-PF) (Alves et al., 2013). However, further studies are needed to better understand these differences and their implications, particularly for *Vibrionaceae* species, due to their importance in aquaculture outbreaks and public health concerns.

Considering that the choice of a PS is one of the most crucial factors for PDI success, studies on *V. parahaemolyticus* in ASW were conducted using two cationic (TMPyP and MB), two neutral (CUR and Ce₆Me) and one negatively charged (TPPS₄) PSs (Figs. 4 and 5). Our findings indicate that the positively charged PSs TMPyP and MB were the most efficient ones, capable of eliminating the bacteria to the detection limit of the method (ca. 8 log CFU.mL⁻¹ reduction, $p < 0.05$). These results are in accordance with the literature, since the presence of phosphatidylethanolamine and phosphatidylglycerol in the outer membrane of Gram-negative bacteria confers a negative charge, posing challenges to the binding of neutral and anionic PS (Almeida et al., 2015; George et al., 2009). As a result, ROS with limited diffusion capability may not effectively reach adequately the bacterial cell structures. In contrast, cationic PS can readily electrostatically bind to the negatively charged lipopolysaccharides of the outer membrane of Gram-negative bacteria, displacing divalent cations and creating gaps in the outer membrane (Almeida et al., 2015; George et al., 2009). Notably, TMPyP demonstrated superior performance, especially at low concentrations (0.5 μM), with shorter treatment times and higher inactivation rates compared to MB. Its enhanced efficacy can be attributed to its chemical properties,

including its fourfold positive charges, facilitating stronger electrostatic interactions with bacterial membranes (Muehler et al., 2022). This was confirmed by PS binding assays since TMPyP presented absorption values on average 1.8 times superior to MB. Additionally, considering that ¹O₂ plays a key role in PDI success, TMPyP's higher ¹O₂ quantum yield (0.74) compared to MB's (0.52) may also contribute to its superior photodynamic efficacy in killing bacterial cells (Wilkinson et al., 1993). A previous study has demonstrated TMPyP activity towards *Vibrio campbellii*, a bacterium that causes luminous vibriosis in marine shrimp hatcheries, in seawater (Malara et al., 2017b). However, achieving bacterial inactivation to the detection limit of the method required 5 h of continuous irradiation at low light irradiance (1.2 mW.cm⁻²) and a total light dose of 21.2 J/cm⁻² for a concentration of 20 μM of TMPyP, or for 24 h at a concentration of 1.0 μM (total light dose of 102 J/cm⁻²). In this study, a similar effect was achieved with *V. anguillarum* in 15 min of white light irradiation (total light dose of 90 J/cm⁻²) in the presence of 5.0 μM of TMPyP, albeit with an irradiance approximately 85 times higher. These results suggest that both low and high light irradiance protocols can activate TMPyP to eradicate pathogenic bacteria in aquaculture ponds. Nevertheless, high irradiance protocols offer faster results, reducing the time-of exposure of farmed fish to the PS.

Although the neutral and anionic PSs showed lower efficiency in bacterial photoinactivation, the neutral PS CUR (5.0 μM) and Ce₆Me (20 μM) achieved reductions exceeding 3 log CFU.mL⁻¹ (> 99.9% bacterial reduction), meeting the threshold to be considered as bactericidal (Koeth, 2023). This effect occurred within the first 15 min for CUR and 60 min for Ce₆Me. However, no further inactivation was observed beyond this initial reduction during the remaining 120 min of treatment. A similar stagnation in the effect of MB at 0.5 μM over time was also observed in this study. Knowing that these PSs are not particularly stable under light exposure, with 55 and 75% absorption decay for Ce₆Me and CUR, respectively (Table S2), their photobleaching during PDI treatments may explain this behavior (Sonia Sabbahi Zoubair Alouini and Jemli, 2010; Melo et al., 2023). The limited photostability of a PS is not necessarily a major drawback for aquaculture studies, as their degradation during irradiation period eliminates the need for their removal after treatments, if the photobleaching products are not toxic for the aquatic organisms. While photobleaching and subsequent PS degradation can impact PS efficiency, the application of successive treatment cycles potentially enhances the treatment effectiveness. It is worth mentioning, that although the negatively charged TPPS₄ at 5.0 μM was less efficient than CUR at the same concentration (3 log CFU.mL⁻¹ reduction), it was still able to cause a bacterial concentration reduction of 2.6 log CFU.mL⁻¹ (99.75%). These results are surprising considering the anionic features of TPPS₄. Previous studies have shown that some destabilizing mediators (e.g. divalent metal cations) can improve the entry of non-cationic porphyrins into Gram-negative bacterial cells by influencing the permeability of the outer membrane (Gsponer et al., 2015; Malara et al., 2017b). In 2016, Heimann and colleagues demonstrated that TPPS₄, at concentrations between 10 and 100 μM, was effective in inactivating a *V. parahaemolyticus* strain below the detection limit of their method in oceanic seawater (Malara et al., 2017b). They attributed this phenomenon to ionic interactions formed by TPPS₄ in seawater, which allow TPPS₄ to extract calcium and magnesium from the bacterial outer membrane, thereby altering the cell's permeability (Malara et al., 2017b). These results suggest that TPPS₄ could be useful for bacteria photoinactivation in saline suspensions, although higher PS concentrations may be needed to achieve a sterilization effect.

Aquaculture systems are predominantly affected by Gram-negative bacteria, which are well known to be less susceptible to PDI treatments compared to other microorganisms (Gamelas et al., 2022; Vieira et al., 2019). Moreover, as confirmed in this study, seawater matrices are more difficult to decontaminate by PDI procedures in comparison with other water matrices. Therefore, to ensure the successful and safe implementation of PDI in aquaculture systems, it is crucial to develop protocols that effectively photoinactivate bacteria at lower PS

concentrations and short irradiation periods. In the last years, the application of the inorganic salt KI has been shown to successfully improve PS activity, acting as a PDI co-adjuvant (Gamelas et al., 2022; Vieira et al., 2019, 2018). The main mechanism underlying this combined effect involves the interaction of KI in solution with the $^1\text{O}_2$ produced by the PS upon irradiation that results in the production of reactive iodine species (RIS): iodine radicals (I_2^\bullet); hydrogen peroxide (H_2O_2) and free iodine/tri-iodide (I_2/I_3^-) (Huang et al., 2017; Vecchio et al., 2015; Vieira et al., 2018). While $^1\text{O}_2$ and I_2^\bullet have short lifetimes in aqueous solutions (ranging from milliseconds to nanoseconds), I_2 have a slower decomposition rate between 0.030 and 2.31 min^{-1} in seawater (Truesdale, 1993). This longer life of I_2 may contribute to its increased antimicrobial activity in comparison with ROS produced by the PS.

Our findings indicate that the application of KI enhances the PDI effectiveness of all tested PS, resulting in higher rates of bacterial photoinactivation in ASW. Additionally, for the most efficient PSs (*TMPyP* and *MB*) the addition of KI enabled a tenfold reduction in PS concentration (from 5.0 to 0.5 μM) and/or treatment time required to achieve this effect (at least by threefold). The enhanced effects of PS when combined with KI are largely influenced by their intrinsic properties. Considering this process is initiated by the reaction of $^1\text{O}_2$ and KI, a high quantum yield of this ROS typically results in the formation of a greater amount of RIS. Our results reflect this trend as *CUR*, the PS with the lowest quantum yield of $^1\text{O}_2$ (0.37 in water) (Gonçalves et al., 2017), was the only one that did not inactivate the bacteria to the detection limit of the methodology ($\sim 8 \log \text{CFU.mL}^{-1}$ reduction) when combined with KI. However, it is also evident that $^1\text{O}_2$ production is not the sole factor influencing this process, as *TPPS₄* and *Ce₆Me*, which have similar singlet oxygen quantum yields (0.62 and 0.64, respectively) (Fernandez et al., 1997), showed very different behaviors in the presence of the KI. *TPPS₄* (at 0.5 μM), which alone had no photodynamic activity towards *V. parahaemolyticus*, caused a significant reduction in bacterial viability to the limit of detection of the method ($\sim 8 \log \text{CFU.mL}^{-1}$ reduction) in the presence of KI (Fig. 5C). On the other hand, *Ce₆Me* required a higher concentration (20 μM) to reach the same inactivation level (Fig. 5B). This difference may be attributed to the distinct physicochemical properties of the PS in water. In fact, while *TPPS₄* is water-soluble and stable under irradiation (Huang et al., 2018), *Ce₆Me*'s hydrophobicity, aggregation behavior in water and low photostability (Table S2) reduce the $^1\text{O}_2$ production during assays (Bresoli-Obach et al., 2021; Sampaio et al., 2020), thereby hindering the formation of the threshold iodine species level necessary for increased bactericidal activity.

Previous reports have highlighted the tremendous potential of KI as PDI co-adjuvant with PSs *in vitro* suspension (PBS), but also have shown that when complex matrices are present, the KI adjuvant effect tends to be nullified (Bartolomeu et al., 2021; Braz et al., 2020; Damrongrungruang et al., 2023; Huang et al., 2018; Vieira et al., 2018). Remarkably, this study represents the first study where PS concentrations below 1.0 μM in the presence of KI were effective in disinfecting seawater matrices (ASW), highlighting KI as a potent adjuvant even when applied in a complex matrix such as seawater. Moreover, our experiments conducted in different suspension matrices revealed that in the presence of KI, the difference in efficiency between PDI treatments in PBS and ASW was less pronounced compared to treatments using the PS alone. These results suggest that the combined treatment with KI could mitigate the reduced efficiency of PDI in suspension media containing high levels of salts, thereby presenting a promising approach for aquaculture applications.

Before considering the direct application of PDI for disinfecting water and equipment in fish farms without harming the animals, further studies are needed to evaluate the toxicity of these treatments on farmed fish. Furthermore, it is also important to consider that the release and accumulation of PS and KI in aquatic environments can potentially cause harmful effects including toxicity to marine life (Iwamoto and Shiraiwa, 2012; Penninks et al., 2017; Zheng et al., 2005). To transpose the PDI for aquaculture applications, it is therefore important to implement some safety procedures to ensure that the amount of PS release to the aquatic

environment do not possess any harmful effect nor the KI salt is used in toxic amounts. One potential strategy for mitigating PS accumulation in the environment is the use of PS that are prone to photobleaching, provided that the resulting photodegradation byproducts are not harmful to aquatic organisms. For instance, photodegradation products of *CUR* and *MB*, the PS used in this study, as well as those from chlorophyllin compounds, have been shown to have minimal harmful effects on marine organisms such as fish, *Daphnia magna* and algae (Al-Baldawi et al., 2018; Bartolomeu et al., 2024; Häder et al., 2016; Heaton and Marangoni, 1996; Lima et al., 2022). However, there is limited information on the long-term accumulation and environmental impact of these degradation products. It is worth noting that the quantities of PS used are in the micromolar range.

Another strategy to prevent PS accumulation in aquatic environments is their immobilization on solid supports for removal after treatment (Spagnul et al., 2015). This strategy has been explored using materials such as magnetic nanoparticles, glass, resins, polymers, and silicones (Alves et al., 2014b; Carvalho et al., 2010; Castro et al., 2017; Gamito et al., 2024; Mesquita et al., 2014; Nyga et al., 2021; Santos et al., 2023). For instance, in 2014, our research group showed that the immobilization of a tricationic porphyrin (*Tri-Py⁺-MePF*) in silica-coated magnetic nanoparticles of Fe_3O_4 not only achieved high efficiency against *Allivibrio fischeri* and *E. coli* but also maintained bactericidal activity after six-cycle reuse (Alves et al., 2014b).

Therefore, immobilization not only facilitates the removal of PSs after treatment, but also enables their reuse in subsequent treatment cycles, thereby reducing costs associated with the PDI process (Alves et al., 2014b; Mesquita et al., 2014). Although no specific immobilization methods for KI have been documented to date, a recent study successfully used a polymeric film entrapping Rose Bengal and iodide anions for PDI applications (López-Fernández et al., 2022). This film efficiently inactivated *Pseudomonas aeruginosa* and *E. coli*, while securely retaining the iodide anions within the matrix, thereby reducing the risk of environmental release. In the future, further detailed research is needed to optimize the immobilization of PSs and potassium iodide on solid supports for aquaculture applications and to evaluate the long-term ecological impacts of these PSs and their degradation products.

Another important aspect for the success of PDI is related to the light source used (Piksa et al., 2023). In this study, we used white light delivered by LED systems, considering that artificial white light sources are already employed in indoor aquaculture facilities to regulate the photoperiod of fish and other farmed organisms (Gillani et al., 2022; Wang et al., 2023). Therefore, utilizing these light sources for PDI treatment would likely be practical and cost-effective. Furthermore, due to the broad wavelength range of white light (400–700 nm), it has the potential to activate PSs with distinct absorption properties. In fact, the total light dose absorbed by each tested PS varied, as calculated according to Schaberle (Schaberle, 2018) (see Fig. S1). *Ce₆Me* exhibited the highest absorption spectrum overlapped with the LED emission followed by *MB*, *CUR*, *TMPyP*, and *TPPS₄* which represent an effective irradiance of respectively 55, 31, 19, 13 and 6 mW.cm^{-2} . However, despite some PSs absorbing significantly less light than others (e.g., *TMPyP* and *TPPS₄*), they are still capable of producing ROS and inactivating bacteria to the detection limit of the method, either alone or with the assistance of KI (Figs. 3–5). Daylight may also be a viable alternative to artificial light sources (Alves et al., 2008; Martins et al., 2018). In fact, this natural light source has several advantages over artificial ones, such as being cost-effective, covering a large area, and providing a fast inactivation rate (Alves et al., 2008). However its irradiance can be inconsistent and its application is limited for outdoor aquaculture systems or facilities specifically designed to allow sunlight to penetrate (Piksa et al., 2023).

5. Conclusion

This study explored the potential of PDI for the photoinactivation of Gram-negative bacteria relevant to the aquaculture sector, namely

V. parahaemolyticus, *V. anguillarum*, and *E. coli*.

Our findings revealed that the cationic PSs, *TMPyP* and *MB*, in PBS, were effective in the photoinactivation of the studied bacteria to the detection limit of the method (~ 8 log reduction of CFU.mL⁻¹) at concentrations ≤ 0.5 μ M. However, tenfold higher *TMPyP* and *MB* concentrations (5.0 μ M) were required to achieve a similar photoinactivation effect in suspensions with a similar composition to ASW. This different photoinactivation profile may be attributed to the presence of high concentrations of ions that can act as scavengers of ROS produced by the PS. Additionally, the bacterial photoinactivation profiles revealed varying susceptibilities among the studied bacteria, with *V. parahaemolyticus* being the most susceptible one, followed by *V. anguillarum* and *E. coli*. These differences appear to be linked to the distinct impact of PDI on the outer membrane of the bacterial cell walls. The combined use of KI and PDI significantly improved the effectiveness of all tested PS in PBS (threefold), and even more in AWS (tenfold), resulting in higher rates of bacterial photoinactivation and shorter treatment times to achieve effective bacterial photoinactivation. These results suggest that KI can effectively counteract the reduced efficacy of PDI in complex matrices, such as in ASW. This highlights KI as a promising strategy for enhancing bacterial inactivation by PDI in aquaculture settings.

Overall, these findings indicate that PDI treatments with different PS in combination with KI constitute a potential approach for the control of bacteria in the aquaculture industry, eliminating effectively both pathogenic agents for farmed fish and consumers.

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Animal and human rights statement

Not applicable (no living animals were used in this study).

CRediT authorship contribution statement

Cátia Vieira: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Maria Bartolomeu:** Writing – review & editing. **Carlos J. P. Monteiro:** Writing – review & editing, Investigation. **Jesús L. Romalde:** Writing – review & editing. **Pedro P. Gallego:** Writing – review & editing. **M. Graça P.M.S. Neves:** Writing – review & editing, Supervision. **M. Amparo F. Faustino:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Adelaide Almeida:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2024.741882>.

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