

# Reducing *Vibrio* load in *Artemia* nauplii using antimicrobial photodynamic therapy: a promising strategy to reduce antibiotic application in shrimp larviculture

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## Summary

We propose antimicrobial photodynamic therapy (aPDT) as an alternative strategy to reduce the use of antibiotics in shrimp larviculture systems. The growth of a multiple antibiotic resistant *Vibrio harveyi* strain was effectively controlled by treating the cells with Rose Bengal and photosensitizing for 30 min using a halogen lamp. This resulted in the death of > 50% of the cells within the first 10 min of exposure and the 50% reduction in the cell wall integrity after 30 min could be attributed to the destruction of outer membrane protein of *V. harveyi* by reactive oxygen intermediates produced during the photosensitization. Further, mesocosm experiments with *V. harveyi* and *Artemia* nauplii demonstrated that in 30 min, the aPDT could kill 78.9% and 91.2% of heterotrophic bacterial and *Vibrio* population respectively. In conclusion, the study demonstrated that aPDT with its rapid action and as yet unreported resistance development possibilities could be a propitious strategy to reduce the use of antibiotics in shrimp larviculture systems and thereby, avoid their hazardous effects on human health and the ecosystem at large.

## Introduction

Antimicrobial photodynamic therapy (aPDT) is an antimicrobial strategy emerging as a propitious alternative to antibiotics in recent years (Wainwright, 1998; 2009; 2010;

Lee *et al.*, 2004; Konopka and Goslinski, 2007; Donnelly *et al.*, 2008; Almeida *et al.*, 2009; Garland *et al.*, 2009; Nakonechny *et al.*, 2010). aPDT is hypothesized to target microorganisms via photosensitized production of reactive oxygen species (Wainwright, 1998; 2010). It has been shown effective, in killing bacterial pathogens isolated from clinical settings (Komerik *et al.*, 2003; O'Riordan *et al.*, 2005; Nakonechny *et al.*, 2010), fishes (Wong *et al.*, 2005; Magaraggia *et al.*, 2006; Almeida *et al.*, 2009) and environmental samples (Wainwright *et al.*, 1998; Jemli *et al.*, 2002; Spesia *et al.*, 2005; Caminos and Durantini, 2006). Various viral, fungal and protozoan pathogens are reportedly amenable to the reactive oxygen species generated by photosensitizers (Casteel *et al.*, 2004; Jori and Brown, 2004; Costa *et al.*, 2008). Reactive oxygen intermediates (ROI) can initiate a cascade of toxic reactions in microorganisms ranging from alterations of cell wall properties by lipid peroxidation and disruption of membrane bound proteins (Humpries and Sweda, 1998; Cabisco *et al.*, 2000; Komerik *et al.*, 2003) to single- and double-stranded breakages of DNA (Sies and Menck, 1992; Sies, 1993). Because aPDT is mediated through ROI that attack a wide range of cellular targets rapidly, the likelihood of developing resistance becomes plausibly remote. However, the type of photosensitizer used and bacterial cell wall structure strongly influences aPDT targets (Ergaieg and Seux, 2009) like poly-L-lysine-chlorine (e6) (pL-ce6) conjugate (Demidova and Hamblin, 2005). The pL-ce6 conjugate replaces the cations in the LPS of Gram-negative bacteria, distorting the outer membrane structure forming a channel. But the same photosensitizer because of its high molecular weight and the resulting inability to penetrate the peptidoglycan layer becomes less effective against Gram-positive bacteria. On the contrary, Rose Bengal (RB) was found to target the cellular constituents via a diffusion controlled process rather than by active binding to the cell (Demidova and Hamblin, 2005).

Broad spectrum activity of aPDT is obligatory in aquaculture, as the aquatic environment is replete with diverse microflora both beneficial and otherwise and, rampant antibiotic application in this sector has raised serious environmental and human health concerns (Spanggaard

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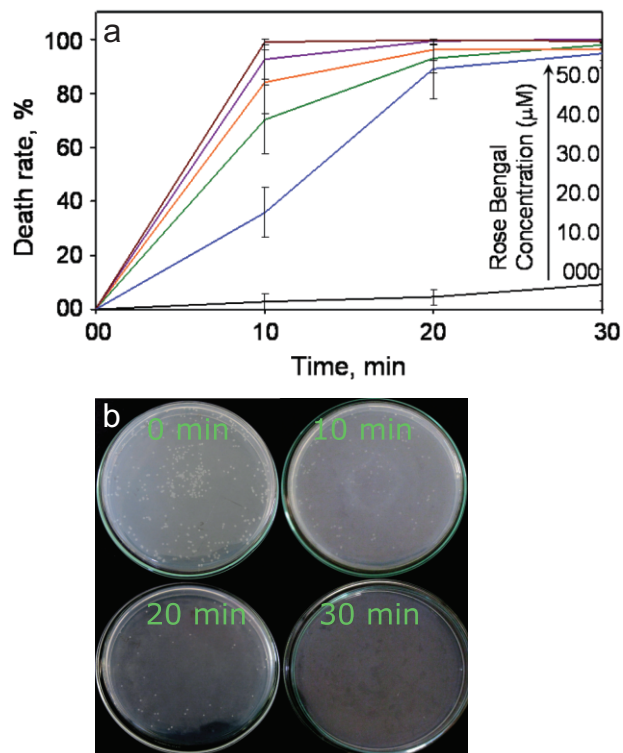
*et al.*, 2001; Alminov, 2009). Multiple antibiotic resistant (MAR) *Vibrio harveyi* has been isolated from shrimp culture systems across Asia and Latin America (Karanasagar *et al.*, 1994; Abraham *et al.*, 1997; Roque *et al.*, 2001). Sixty percent of *Vibrio* isolated from *Artemia* nauplii reared in penaeid hatchery in India were resistant to erythromycin, nitrofurazone and oxytetracycline (Hameed and Balasubramanian, 2000). Besides, there exists a strong evidence towards the transfer of antibiotic resistance genes from the fish pathogen *Aeromonas* to human pathogen *Escherichia coli* (Rhodes *et al.*, 2000a,b; Cabello, 2006; Sorum, 2006) and quinolone resistant gene from aquatic bacteria *Shewanella algae* and *Vibrio* to human Gram-negative pathogens (Jacoby, 2005; Poirel *et al.*, 2005; Robicsek *et al.*, 2005; 2006; Cabello, 2006). Ostensibly, the antibiotic resistant genes of *Vibrio cholerae* associated with the 1992 Latin American cholera epidemic were passed on from antibiotic resistant bacteria that emerged from large-scale antibiotic use by the Ecuadorian shrimp industry (Weber *et al.*, 1994; Angulo *et al.*, 2004).

Over two decades of research has yielded a plethora of alternative methods to replace antibiotic use in aquaculture systems (Verschuere *et al.*, 2000a; Defoirdt *et al.*, 2007). These strategies include pre-empting the host immune system using immunostimulants and interfering with the pathogen directly employing probiotics (Merchie *et al.*, 1997; Verschuere *et al.*, 2000a,b; Defoirdt *et al.*, 2007; Anas *et al.*, 2009; Pai *et al.*, 2010). Despite *in vitro* successes with these strategies, their widespread application is limited by their inconsistency in the field and more importantly their handicap to provide protection during emergencies (Spanggaard *et al.*, 2001; Pai *et al.*, 2010). Emergency situations warrant strategies that directly target and reduce pathogen concentration in the system within a short time. Therefore, despite negative impacts, aquaculturists continue to use antibiotics owing to the lack of viable alternatives with rapid action to combat sudden disease outbreaks/emergencies. To address this issue, we explored the suitability of using aPDT in shrimp culture systems, as a non-toxic and rapid alternative to antibiotics in controlling the *Vibrio* population. The efficiency of photodynamic therapy in controlling the growth of multiple antibiotic resistant *V. harveyi* strain was investigated under both *in vitro* and *in vivo* conditions using RB as the photosensitizer in an *Artemia* nauplii model. Also the targets of aPDT in *V. harveyi* were investigated using SDS and comet assay.

## Results

### Photosensitized killing of *V. harveyi* by RB

We observed a time-dependent increase in the death rate of *V. harveyi* at increasing concentration of RB (Fig. 1A).

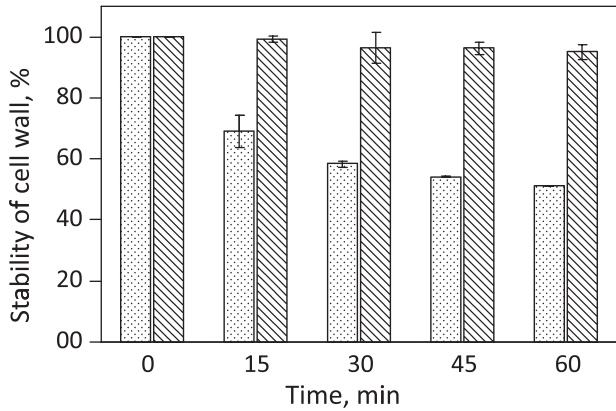


**Fig. 1.** A. Death rate of *V. harveyi* at increasing concentrations of photosensitizer and exposure time. Values expressed as average  $\pm$  SE. No significant differences between the death rates at various concentrations ( $P > 0.05$ ). B. Growth of *V. harveyi* after photoexcitation in the presence of 30  $\mu$ M RB for an incubation period of 0, 10, 20 and 30 min respectively (representative plates).

Interestingly, > 50% death occurred within the first 10 min of photo excitation at 20–50  $\mu$ M concentrations RB, peaking to 80–100% over 30 min. Figure 1B shows the representative images of the responses of *V. harveyi* to photoexcitation in the presence of 30  $\mu$ M RB. Complete elimination of *V. harveyi* was achieved at 30  $\mu$ M concentration of RB after 30 min photoexcitation. Less than 20% death of *V. harveyi* was observed at the highest concentration (50  $\mu$ M) of RB alone (Fig. S1) or when exposed to light without photosensitizer.

### Effect of aPDT on cell wall integrity and genetic stability of *V. harveyi*

aPDT affected both cell wall integrity and outer membrane protein profile (Omp) of *V. harveyi* following photosensitization. We observed a 50% decrease in the cell wall integrity of *V. harveyi* after 30 min of photosensitization (Fig. 2). A complete disintegration was observed in the outer membrane protein profile of *V. harveyi* after photosensitization. While there were three distinct bands between 29–43 kDa typical of *V. harveyi* (Abdallah *et al.*, 2009) in the SDS-PAGE of OmPs extracted from *V. har-*



**Fig. 2.** Cell wall integrity of *V. harveyi* upon photoexcitation in the absence (bar with line) and presence (bar filled dots) of RB. Values expressed as average  $\pm$  SD.

*veyi* before photosensitization, there were none after (Fig. 3A). Biochemical measurements further supported the disruption in the outer membrane integrity by showing a drastic decline in Omp (Fig. 3B).

We assessed DNA strand integrity of *V. harveyi* after photosensitization by single cell gel electrophoresis (comet assay). Surprisingly, the aPDT treatment regime employed in the study did not seem to affect DNA integrity of *V. harveyi* (Fig. 4). The extent of damage was assessed by classifying comets based on their tail lengths: as low or no damage (0–10 mm length), medium (10–20 mm) and heavy (> 20 mm). *V. harveyi* treated with 50 mM cadmium chloride was used as positive control. We observed that the photosensitization in the presence of 30  $\mu$ M of RB did not induce any significant adverse affect to the genetic stability *V. harveyi*.

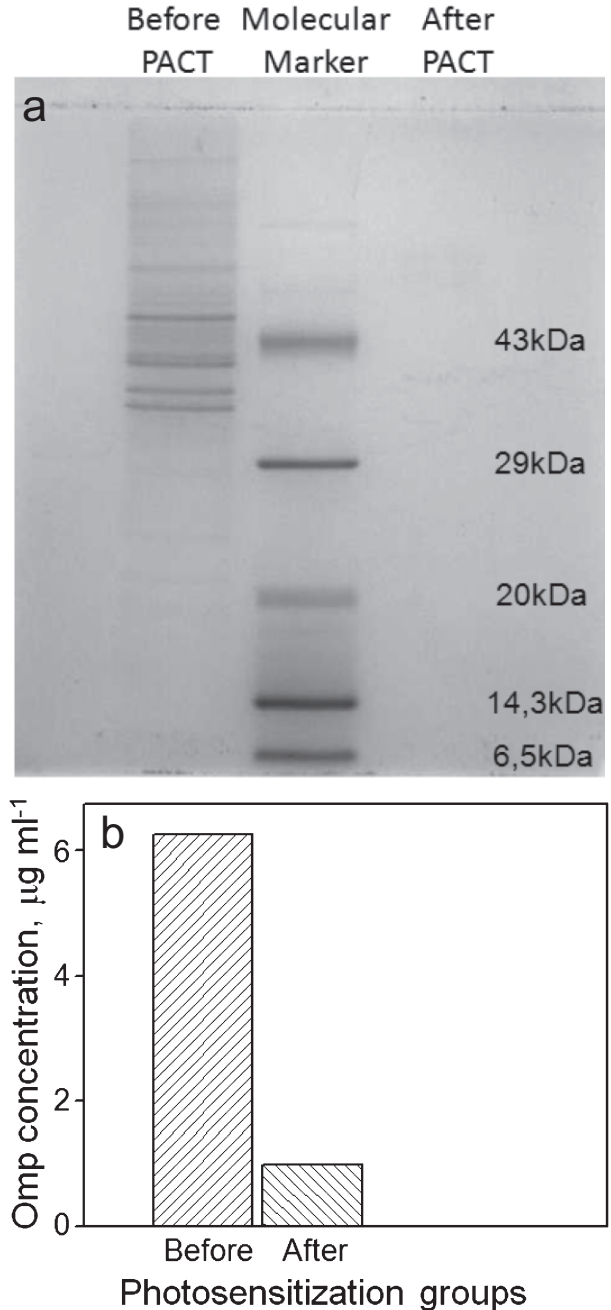
*aPDT is non-toxic to Artemia and accords protection from the pathogen V. harveyi*

We first assessed the toxicity of RB with or without photosensitization in *Artemia* nauplii. The freshly hatched nauplii were exposed to different concentrations of RB for 180 min in the presence and absence of light. The nauplii were considered dead if no appendage movement was observed and survival was calculated from the average number of live nauplii per total count. We found that RB at all concentrations, in the presence or absence of light is not toxic to *Artemia* nauplii (Fig. 5). Separately, when the nauplii were exposed to the pathogen, treated with RB and photosensitized, total bacterial population significantly reduced, especially those of vibrios (Fig. 6). It was observed that, 78.9% of the total heterotrophic bacterial population was reduced in *Artemia* nauplii exposed to aPDT. More interestingly, 91.2% of the *Vibrio* populations were removed from *Artemia* nauplii on aPDT. This indi-

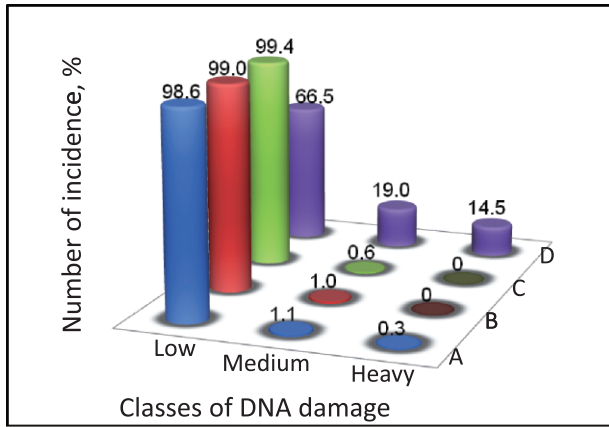
cated that aPDT was effective in killing the pathogen under *in vivo* conditions as well, without deleterious effects on *Artemia* nauplii.

**Discussion**

Continuous evolution of bacterial pathogens demands a constant search for novel strategies to counter their threat

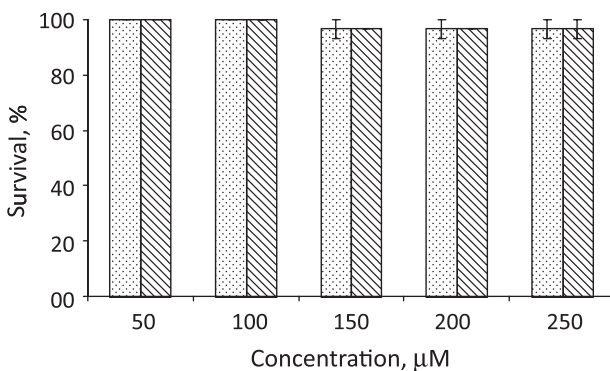


**Fig. 3.** SDS-PAGE images of profile (A) and concentration (B) of outer membrane proteins of *V. harveyi* cells before and after photosensitization with RB for 30 min.



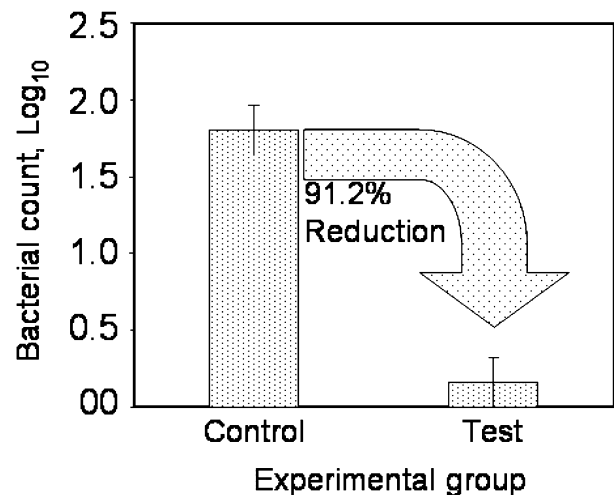
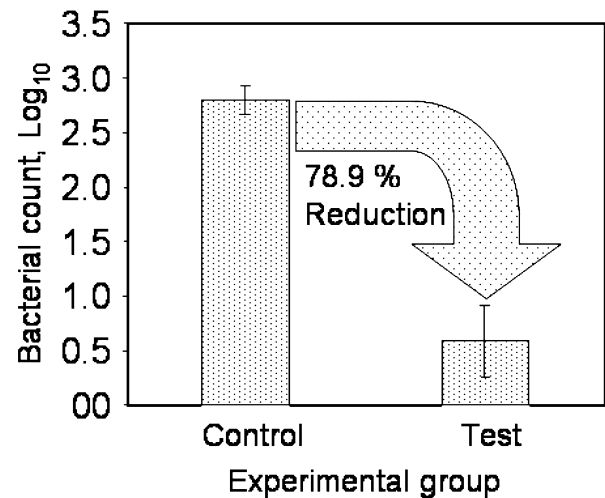
**Fig. 4.** Histogram of comet assay results of *V. harveyi* exposed to photodynamic antimicrobial chemotherapy. Different treatment groups are *V. harveyi* with RB incubated under dark (A); *V. harveyi* exposed to light for 30 min (B); *V. harveyi* after APDT for 30 min (C) and positive controls (D). Comets are classified based on tail length into Low (0–10 mm), Medium (10–20 mm) and Heavy (20–30 mm).

to humans and animals alike. In this study we explored aPDT as an alternative strategy to counter pathogen infestations in shrimp larval rearing systems using a *V. harveyi*-*Artemia* model. A commonly available inexpensive photosensitizer, RB was employed and its efficacy studied against a pathogenic, multiple antibiotic resistant *V. harveyi* strain. RB was selected as the photosensitizer based on its absorption in visible region, water solubility, high singlet oxygen quantum yield and inexpensiveness (Chang *et al.*, 2008). Although, Gram-negative bacteria are more susceptible to the cationic photosensitizer methylene blue, its high toxicity to *Artemia salina* even in the absence of light precludes its biological use (Peloi *et al.*, 2008). *Artemia* nauplii are widely used as live feed in shrimp larval rearing systems, and reportedly an important route of pathogen entry (Triantaphyllidis *et al.*, 1998;



**Fig. 5.** Survival of *Artemia* nauplii after exposure to different concentration of RB in the absence (bar filled with dots) and presence (Bar with slanting line) of light for 180 min. Values expressed as average  $\pm$  SE.

Lavens and Sorgeloos, 2000; Sorgeloos *et al.*, 2001; Vaseeharan and Ramasamy, 2003). Therefore, producing pathogen free *Artemia* without antibiotic administration would plug not only a major pathogen entry point but also prevent antibiotic use in shrimp larval rearing systems. We observed that a photoexcitation for 30 min in the presence of 30  $\mu$ M concentration of RB reduced the pathogen load without inducing toxicity to *Artemia* nauplii (Fig. 1) and the efficiency of aPDT increases with time of photoexcitation. Agreeably, an  $\epsilon$  of 50% was achieved within the first 10 min of photosensitization of RB treated bacteria except, in those treated with 10  $\mu$ M concentration. In contrast, both the highest concentrations of photosensitizer (RB) used or light alone independently could only induce a death rate of < 20%. This confirmed that the bacterial mortality obtained was indeed due to the photosensitizing effect of RB. Because total bacterial removal from the system may be detrimental to the remineralization capaci-



**Fig. 6.** Total heterotrophic bacteria (A) and vibrio (B) population in *Artemia* nauplii, before (control) and after (test) photodynamic antimicrobial chemotherapy.



ties of a closed system (Riemann and Azam, 2002), the achievement of pathogen reduction at 30  $\mu\text{M}$  concentration assumes significance. It may also be noted that RB at lower concentrations is non-toxic, evidenced by its use in ophthalmologic diagnosis (Paugh, 2008). In contrast, its higher concentrations can induce both apoptotic and non-apoptotic cell deaths in different melanoma cells (Mousavi and Hersey, 2007) and inhibit P-glycoprotein activities (Mizutani, 2009). Here in our observations, RB at 30  $\mu\text{M}$  concentration resulted in > 80% death rate within 10 min. Such a reduction in bacterial load should be sufficient to prevent larval infections in hatcheries without overtly unbalancing the larval mesocosm.

To shed light on the targets of aPDT on *V. harveyi*, we used SDS and single cell gel electrophoresis assay (Comet assay). The continuous monitoring of incubation temperature ( $28 \pm 1^\circ\text{C}$ ) during the 30 min photoexcitation ruled out possible thermal shock. In the SDS assay, the effect of aPDT on bacterial cell wall integrity is represented as a function of their sensitivity to detergent mediated cell lysis. The outer membrane if already damaged, when treated with a detergent such as sodium lauryl sulfate, will show a sharp decline in the optical density ( $\text{OD}_{600}$ ) due to leakage of cell content resulting in bacterial death (Lok *et al.*, 2006). In the absence of any membrane damage, the optical density will not show sharp changes.

We confirmed that the aPDT principally impacts the cell wall integrity of *V. harveyi*. The cell wall integrity of *V. harveyi* decreased by 50% in 30 min of aPDT. This effect was confirmed by extracting and quantifying the outer membrane proteins (Omps) of *V. harveyi*. Omps are located on the external surface of Gram-negative bacteria in tight association with the peptidoglycan layer and play a key role in bacterial adaptation to environmental changes. The protein bands specific for Omps appeared in the SDS-PAGE of untreated *V. harveyi* cells, whereas the aPDT-treated cells did not yield any specific bands because of the drastic reduction in the concentration of Omps in these cells. The Omp concentration of control and aPDT-treated samples were found to be 6.25 and 1  $\mu\text{g ml}^{-1}$  respectively. Omps damage extends the access of the photosensitizer, Toluidine blue, to inner membrane, making it more destructive (Sahu *et al.*, 2009). Sahu and colleagues (2009) suggested the possibility of total destruction of the cell wall including the inner membrane of *E. coli* on aPDT (Sahu *et al.*, 2009). They reported the appearance of deep grooves ( $\sim 200$  nm) on the surface of *E. coli* at higher light doses leading to the leakage of the intracellular contents and concomitant cell flattening. This also correlates with the decreased absorbance in SDS assay. Studies by Tegos and Hamblin (2006) have demonstrated that RB and light probably kill bacteria by generating extracellular singlet oxygen that destroys the membrane from the outside in.

Recently, Jin and colleagues (2010) demonstrated the photosensitized damage of cell wall of Gram-negative and Gram-positive bacteria using the photosensitizer haematoporphyrin monomethyl ether by atomic force microscopy.

Rose Bengal used in the present study is a standard photosensitizer, coming under the xanthene group of dyes (Wainwright, 1998). In standard photodynamic reactions, RB molecules absorb 450–580 nm wavelength light from a halogen lamp and are excited into a singlet state ( $^1\text{RB}$ ) with around  $10^{-8}$  s lifetime. The excited molecules transfer energy to molecular oxygen ( $^3\text{O}_2$ ) present in the proximity, leading to the generation of singlet oxygen ( $^1\text{O}_2$ ) and initiate a cascade of energy transfer reactions and intermediates, which are collectively named as ROI (Banks *et al.*, 1985). ROI induce a series of toxic reactions in living cells, including DNA damage and breakage, lipid and protein denaturation (Maisch *et al.*, 2005). RB being an exogenous free dye enters the cell by diffusion-controlled process rather than by active binding to the cell (Demidova and Hamblin, 2005). Therefore, the primary site of action of RB is the cell surface rather than the genetic material of *V. harveyi*. This was confirmed with comet assay result, where we observed neither medium nor heavy damage of DNA in aPDT-treated cells. Reactive oxygen species, the main destructive force in aPDT, has a life-time of  $\sim 1\text{--}3$   $\mu\text{s}$  in water,  $\sim 100$  ns in lipid regions of cell membrane, and  $\sim 250$  ns in cytoplasm (Pandey and Zheng, 2000). Therefore, the diffusion range of singlet oxygen is predicted to be limited to approximately 45 nm in cellular media (Pandey and Zheng, 2000). This short lifetime and narrow diffusion range do not permit the ROI generated on the surface of bacteria to interact/destroy the genetic material of *V. harveyi*. Therefore, we attribute the photodynamic killing of *V. harveyi* to the cell wall disintegration by the reactive oxygen species produced upon photoexcitation of RB.

We evaluated the possibility of reducing bacterial pathogen entry into shrimp larval rearing systems by subjecting *Artemia* nauplii, a principle entry route of pathogens, to aPDT. *Artemia*, a crustacean, is a major live feed organism particularly in shrimp larval rearing. We exposed *Artemia* nauplii challenged with *V. harveyi* to aPDT and observed that the *Vibrio* population in aPDT-treated animals reduced by  $\sim 91\%$  in 30 min. It may be noted that while *Vibrio* spp. are pathogenic to shrimp larvae, their absence too can negatively impacts larval survival because they perform vital role in the transformation of organic matter (Pruzzo *et al.*, 2005). The photodynamic therapy described here reduces the total heterotrophic bacterial load of the system whereby making it a promising application in the closed shrimp larval rearing system. We believe that the rapid removal of pathogens is most important in larval rearing systems of shrimps as total

mortality of these animals may set-in within a short time of infection (Chen, 1992). Although many alternative strategies such as probiotics, immunostimulants and water quality management aim to restrict *Vibrio* population in larval rearing systems, all fail to respond to infections that have already set-in forcing aquaculturists to depend on antibiotics for immediate resolution. However, the deleterious effects of residual antibiotics on humans and environment are more alarming and require immediate attention. Many countries have already banned/restricted the application of antibiotics in aquaculture and multilevel monitoring of the produce for antibiotic residues prevents antibiotic-containing products from reaching consumers (Johnston and Santillo, 2002).

In this backdrop, the present study proposes the introduction of aPDT as an alternative strategy for negating/minimizing the use of antibiotics in shrimp larval rearing systems to control disease outbreaks. The advantages of aPDT, compared with the other alternative disease control strategies such as probiotics or immunostimulants, are its fast action and virtually no possibility of resistance development. Recurrence of bacterial diseases in high-volume commercial tanks demands repetitive applications of excessive antibiotics. Maybe the high-volume larval rearing tanks complicate the use of aPDT in its present capacity; nevertheless, the technique can easily be adapted when animals are being transferred from one tank to another, when volumes are considerably lower. During the transfer process, the larvae can be kept in small volumes of water for 30–60 min and exposed to aPDT. The RB containing water then becomes amenable to small scale biological or chemical treatments before discharge (Parham *et al.*, 2011). aPDT could be very useful in the prevention of infections occurring through live feed such as *Artemia*, which can be treated in this manner before feeding. aPDT could also aid in the lowering of bacterial load of post larvae for transport to distant grow-out farms.

## Experimental procedures

### *Bacteria, Artemia and Photoexcitation conditions*

*Vibrio harveyi* (MCCB 111) was obtained from the culture collection of National Centre for Aquatic Animal Health (NCAAH), Cochin University of Science and Technology, Cochin India. The strain was previously isolated from moribund *Penaeus monodon* larvae. *V. harveyi* was inoculated into ZoBell's marine broth prepared in 15 gl<sup>-1</sup> seawater and grown overnight at 28 ± 1°C on a shaker at 120 r.p.m. Working cultures of *V. harveyi* was maintained on ZoBell's marine agar slants, subcultured every 2–3 weeks. The purity of the culture was confirmed by Gram staining and biochemical tests.

Gnotobiotic *Artemia* was prepared following the method of Sorgeloos and colleagues (1977). *Artemia* cysts (0.5 g) were

hydrated in 45 ml tap water and kept in continuous suspension by sparging filter sterilized air from bottom. All materials used for rearing gnotobiotic artemia were previously sterilized. After 1 h, the suspension was diluted with an equal volume of sodium hypochlorite and incubated for 5–7 min. The decapsulated cysts were filtered through 100 µ sieve and washed thoroughly with sterile seawater to remove residual chlorine. *Artemia* nauplii thus reared were collected in a 120 µ sieve, washed thoroughly with sterile seawater, placed in a Petri dish with 10 ml seawater and treated with chloramphenicol (1 gl<sup>-1</sup>) for 30 min.

All samples for photoexcitation were placed under a 150 W halogen lamp with an output spectrum ranging from 450 to 600 nm. Samples (bacteria or artemia) were maintained in borosilicate glass vials with PTFE-lined caps at a distance of 50 cm from the halogen lamp and shaken intermittently to negate any heating effect.

### *In vitro experiments*

**Bacterial killing assay.** Borosilicate glass vials were filled with 10<sup>6</sup> cfu ml<sup>-1</sup> of *V. harveyi* in 2 ml PBS (pH 7.3 ± 0.2) and 0–50 µM concentrations of RB in triplicate. Light controls were devoid of RB while dark controls were one set of all combinations aluminium foil double-wrapped. The efficiency of photodynamic antimicrobial chemotherapy was expressed as percent death rate (Zheng and Zhu, 2003). One hundred microlitres of sample was retrieved from all vials at 10 min intervals and spread over the surface of ZoBell's marine agar plates in duplicates, incubated at 28 ± 1°C for 24 h before enumerating the total number of colonies. Death rate ε was calculated in comparison with light control using the following equation (Zheng and Zhu, 2003).

$$\text{Death rate \% } (\epsilon) = \frac{(N_1 - N_2) \times 100}{N_1}$$

Where, N<sub>1</sub> and N<sub>2</sub> are the number of colonies developed on the control and experimental plate respectively.

**Effect of aPDT on cell wall integrity.** The effect of aPDT on cell wall integrity of *V. harveyi* was investigated by SDS assay following the method of Lok and colleagues (2006). The assay was repeated three times with duplicate tubes at each time. Briefly, overnight cultures of *V. harveyi* cells were washed copiously with sterile phosphate-buffered saline (8 g NaCl, 0.2 g KCl and 1.44 g KH<sub>2</sub>PO<sub>4</sub> prepared in distilled water 1 l, pH 7.4) and dispensed in sterile quartz cuvettes to a volume of 2 ml. After measuring the initial absorbance, the test solution was supplemented with RB (30 µM) and SDS (0.1%). A control cuvette without RB was maintained. The cuvettes were exposed to light for 60 min and the absorbance at 600 nm was recorded at every 15 min. The percentage decrease in absorbance compared with the initial reading was plotted against time.

The outer membrane protein profile of control and aPDT-treated *V. harveyi* cells were evaluated following standard protocols (Laemmli, 1970; Sanderma and Stroming, 1972). The outer membrane protein was extracted according to Sabri and colleagues (2000) and Deb and colleagues (1995) with slight modifications. The control and aPDT-treated cells

of *V. harveyi* were washed copiously with sterile saline and resuspended in PBS containing 5 mM phenylmethylsulfonyl fluoride (PMSF). Cells were disrupted in an ultrasound sonicator by a number of 40 s pulses until the suspension became translucent. Unbroken cells and cellular debris were removed by centrifugation at 5000 *g* for 20 min. The pellet discarded, the supernatant collected was centrifuged at 100 000 *g* in an ultracentrifuge (Beckman Coulter) for 40 min at 4°C. The supernatant discarded, the pellet was resuspended in 2 vol of 2% (w/v) SDS, incubated at room temperature for 1 h and centrifuged at 100 000 *g* for 40 min at 4°C. This pellet was resuspended in saline and stored at -20°C until use. Outer membrane proteins (Omp) were analysed by SDS-PAGE (Laemmli, 1970) with 15% acrylamide in the separating gel and 5% acrylamide in the stacking gel. The proteins were visualized by staining with 0.2% Coomassie Brilliant Blue G250. The Omp concentration was quantified against BSA as the standard using a spectrophotometer (Sanderma and Stroming, 1972).

**Effect of aPDT on genetic stability.** The effect of aPDT on the stability of genetic material was investigated by comet assay following the method of Singh and colleagues (1999). Briefly, 10<sup>6</sup> bacterial cells were mixed with 500 µl of 0.5% low melting point agarose prepared in TAE buffer containing RNase (5 µg ml<sup>-1</sup>), SDS (10%) and lysozyme (0.5 mg ml<sup>-1</sup>). Bacterial cells impregnated in agarose solution were layered over a microscopic slide pre-coated with a thin film of 0.5% agarose solidified by incubating at 4°C for 30 min. Slides were then incubated at 37°C for 30 min and cells lysed by immersing in lysis solution containing NaCl (0.15 M), Tris (10 mM, pH 10), EDTA (100 mM), Triton × 100 (1%) for 1 h at 37°C. The slides were again immersed in a solution containing NaCl (0.15 M), Tris (10 mM, pH 7.4), and EDTA (10 mM) and proteinase K (1 mg ml<sup>-1</sup>) for 2 h at 37°C to digest cellular proteins. The slides were equilibrated with 300 mM sodium acetate and electrophoresed at 50 V for 15–20 min. Following electrophoresis, the slides were immersed in 1 M ammonium acetate in ethanol for 30 min, then in absolute ethanol for 1 h, air dried at 25°C, immersed in 70% ethanol for 30 min and air dried. Slides were stained with 1 ml freshly prepared solution of 1 µl SyBr green in TE buffer. The comets were observed under a fluorescent microscope (Olympus BX 51) equipped with dichroic filter pairs (Excitation filter: 470–490 nm, Emission filter 520 nm, Dichroic 500 nm) and a CCD camera. The comet length was measured and processed using the software Image Pro. The nucleic acids were classified based on comet length as low or no damage (0–10 mm length), medium (10–20 mm) and heavy (> 20 mm) damaged groups and expressed as % number of incidence in the histogram. The assay was repeated three times with duplicate tubes at each time.

$$\text{Number of incidence, \%} = \frac{\text{Number of cells in each class}}{\text{Total cells counted}} \times 100$$

#### In vivo experiments

**Evaluation of the toxicity of RB to Artemia nauplii.** Gnotobiotic *Artemia* prepared as mentioned earlier were dispensed in

the wells of a 24-well microplate containing 2 ml sterile seawater at a density of 10 animals per well. RB in 0–250 µM concentrations range was added to the wells and the plate exposed to light for 180 min. A set of each was maintained without exposure to light as dark control. Following the incubation, differential counts of live and dead *Artemia* nauplii were recorded. All the combinations were in quadruplicate.

**Efficiency of aPDT in eliminating *V. harveyi* from Artemia nauplii rearing system.** Ten individuals of gnotobiotic *Artemia* were deposited in Petri dishes filled with 10 ml seawater containing 10<sup>5</sup> cfu ml<sup>-1</sup> *V. harveyi* and incubated for 3 h following which the animals were washed with sterile seawater and divided into two groups designated as control and test. The test groups were subjected for aPDT by exposing to light for 30 min in the presence of RB (30 µM). Both the groups were transferred into microcentrifuge tubes and homogenized with a micropestle for evaluating the total heterotrophic bacteria and *Vibrio* populations. One hundred microlitres of the homogenate was plated on ZoBell's marine and TCBS agar plates respectively and colonies counted after 24 h incubation at 28°C. The experiments were repeated thrice. Smooth, opaque and thin-edged fermentative or non-fermentative colonies on TCBS agar were picked and confirmed as *Vibrio* by assessing cytochrome *c* oxidase activity, glucose dissimilation and sensitivity to the vibriostatic agent 2,2-diamine-6,7-diisopropylpteridine phosphate (O/129). *Pseudomonas* gives oxidation reaction to glucose while *Aeromonas* and *Vibrio* are fermentative. *Aeromonas* spp. are generally resistant to O/129 while *Vibrio* are sensitive.

#### Statistical analysis

Differences in the death rate of *V. harveyi* on photoexcitation with different concentrations of RB were analysed using one way ANOVA. A significance level  $P < 0.05$  was considered (Bailey, 1995).

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### Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Death rate of *V. harveyi* at increasing concentrations of photosensitizer alone at different time intervals.

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