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**Photodynamic Antimicrobial
Chemotherapy for Pathogenic *Vibrio*
Control in Prawn Hatcheries**

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In the College of Science and Engineering

James Cook University

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Research conducted in this thesis comply with all ethic measures established by James Cook University and outlined by James Cook University Ethic Committee.

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Statement of the Contribution of Others

This thesis includes collaborative work with my supervisors, Associate Professor Kirsten Heimann (JCU), Associate Professor Michael Oelgemöller (JCU) and Dr. Lone Høj (AIMS). While the data collection was principally conducted by Danilo Malara, experimental design, data interpretation, data analysis, developing the approach and arguments as well as multiple manuscript drafts, were performed by myself, Danilo Malara, in collaboration with all of my advisory panel, as described in Table SCO.1. The research conducted in this thesis complies with all ethic measures established by James Cook University and outlined by James Cook University Ethic Committee.

Table SCO.1: Contribution to thesis chapter and experimentation. DM= Danilo Malara, LH = Dr. Lone Høj, KH = A/Professor Kirsten Heimann, MO = A/Professor Michael Oelgemöller.

Chapter n.	Experimental design	Data collection	Data analysis and interpretation	Approach and arguments	Manuscript draft
2	DM, LH	DM	DM, LH, KH	DM, LH, KH,	DM, LH, KH, MO
3	DM, LH, KH	DM	DM, LH, KH	DM, LH, KH	DM, LH, KH, MO
4	DM, LH, KH	DM	DM, LH, KH	DM, LH, KH	DM, LH, KH, MO
5	DM, LH	DM	DM, LH, KH	DM, LH, KH	DM, LH, KH, MO

Abstract

In the last two decades, the aquaculture industry has grown significantly, providing fish, molluscs and crustaceans to the global market. Microbial pathogens are the principal cause of massive financial losses in many fish and crustacean farms. Disinfection using ozone, chlorine or UV irradiation are expensive and leave toxic by-products. Biosecurity threats through the introduction of wild brood-stock and live feed is a major concern for the aquaculture industry. Microalgae and/or *Artemia* nauplii (brine shrimp), have been identified as potential vectors for microbial pathogens that can result in high mortalities, particularly in hatcheries. Bacteria can proliferate rapidly in intensive aquaculture productions due to the high density of target animals, live feed organisms and build-up of biological waste that generate ideal conditions for many potential pathogens. The current inability to bio-secure farmed aquaculture animals through appropriate disinfection strategies of live feed without altering the quality of the final product is an area requiring urgent research, as it is the main barrier to cost-effective product development; the main hurdle to competitive expansions into national and international markets.

Recently, photodynamic antimicrobial chemotherapy (PACT) has emerged as a promising water sterilisation technique. PACT uses the activation of photosensitisers by light to generate highly reactive oxygen species (ROS), which indiscriminately oxidises cell wall and cell membrane components (i.e. lipids, proteins, carbohydrates). After entry of the photosensitiser into cells, produced singlet oxygen also targets intracellular components such as organelles, membrane compartments, and nucleic acids (i.e. DNA and RNA). Due to the indiscriminate action of $^1\text{O}_2$, resistance cannot develop, unlike in the case of antibiotics. The choice of photosensitiser is crucial, and compounds that photobleach with time are preferred for aquaculture purposes to avoid build up in water or the farmed animal. Porphyrins are a group of natural or synthetic compounds that fit the photobleaching requirement, but to date no research has specifically investigated porphyrin-based sterilisation efficiency in prawn hatcheries. In addition, the porphyrin-based antimicrobial efficiency for the ones used in this research has not been tested against luminescent virulent strains of *Vibrio*

harveyi-related species, the main causative agents for luminescent vibriosis, which causes large losses for finfish, bivalves and prawn industries.

Each chapter of this thesis addresses specific objectives that, in conjunction with the other chapters, contribute to the principal aim of the research: investigate the potential of using PACT to control *Vibrio* bacteria in aquaculture. Chapter 2 details objectives relating to method development to select a suitable *Vibrio harveyi*-related strain to be used in the following chapters.

While Chapter 1 reviews PACT history and its application with special interest to aquaculture, the method development chapter (chapter 2), pathogenicity towards prawns after injection and luminescence intensity of two selected *Vibrio* strains were evaluated. Challenge experiments were performed with *Penaeus monodon* and Koch's postulates were fulfilled. Precise identification was obtained using molecular techniques including multiplex PCR, housekeeping gene analysis and construction of a phylogenetic tree. The strongly luminescent *Vibrio sp* ISO7, was demonstrated to be highly virulent towards *P. monodon*, killing 100% of the injected animals and molecular techniques revealed that this species belongs to the *V. campbellii* group. In contrast, *V. owensii* 47666-1, previously described as a luminescent prawn larvae pathogen, caused only 25% mortality in the first challenge experiment. Pathogenicity was regained in bacteria re-isolated from sick prawns, with 100% mortality of *P. monodon* obtained in a second challenge experiment, however the strain was found to be not luminescent. Hence, *V. campbellii* ISO7 was selected as the model bacterium based on its high virulence after injection and strong luminescence signal, making it suitable for the development of a fast luminescence-based assay to determine the efficiency of different porphyrin treatment protocols.

In chapter 3, the objectives were 1) to compare the photostability of the tetra-cationic 5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (p-toluenesulfonate) [TMPyP] and the tetra-anionic 4,4',4'',4'''-(porphine-5,10,15,20-tetrayl) tetrakis (benzenesulfonic acid) tetrasodium salt hydrate [TPPS₄] porphyrins in seawater to confirm their suitability for aquaculture purposes and 2) to investigate the capacity of TMPyP and TPPS₄ to inactivate the model bacterium *Vibrio campbellii* ISO7.

For the first objective, the photostability of the cationic TMPyP and the anionic TPPS₄ porphyrins were investigated by recording their full light spectra between 350 and 750 nm after irradiating seawater containing different porphyrin concentrations (1, 5, 10 and 20 µM) of porphyrins with high power 150 W LED lights. Results obtained using the maximum peak of absorbance for each porphyrin, showed porphyrin degradation (photobleaching) for both the cationic and the anionic porphyrin within 24 h of irradiation. In addition, the dark control (samples not irradiated) did not photobleach, confirming that porphyrin degradation was due to exposure to light. The results confirmed that both porphyrins were good candidates for aquaculture applications based on their photobleaching properties showing relatively fast degradation (within 24-h at concentration of 1 µM and more than ¼ of absorbance reduction at the concentration of 20 µM).

Antimicrobial activity of the porphyrins was investigated using dose-response and time-course experiments. Twenty µM of the cationic porphyrin achieved 100% lethality in the model bacterium (start concentration of *V. campbellii* ISO7 ~10⁷ CFU·mL⁻¹) after five hours, which was validated using biological activity (luminescence), growth experiments (CFU, absorbance) and 7-day regrowth experiments. Consistent with previous reports, the anionic photosensitizer did not achieve inactivation of the model bacterium and was therefore not investigated further. As demonstrated, water sterilization was achieved after between five and twelve hours depending on the concentration of the cationic porphyrin; however, the effectiveness of sterilization in mixed culture with live feed organisms still needed to be confirmed.

In chapters 4 and 5, the potential of using the TMPyP porphyrin to reduce bacteria loads of microalgae cultures (free of the model bacterium) and *Artemia* cysts was investigated including an evaluation of possible toxic effects caused by singlet oxygen generated during PACT towards the live feed organisms themselves. In chapter 4, the viability of microalgae cells was first evaluated using flow cytometry based on chlorophyll fluorescence and the live-dead stain Propidium Iodide (PI) during a six-hour dose-response treatment with the porphyrin. The treatment time was chosen based on bacterial disinfection results shown in chapter 3. Sensitivity to ¹O₂ was species-specific and related to cell wall characteristics. Of the five different microalgae used,

only *Nannochloropsis oculata* was highly resilient to the six-hour treatment with up to 50 μM of the cationic porphyrin. The results were unexpected, as photosynthetic microorganisms possess detoxification systems for $^1\text{O}_2$ and other reactive oxygen species (ROS). However, $^1\text{O}_2$ produced externally to the cell may be able to photooxygenate and destabilize cell membrane components, leading to cell death. The thick cell wall of *N. oculata* most likely protected the cell membrane from fast photooxidation. The highly resilient microalga, *N. oculata* was therefore used in mixed culture with the model pathogen and treated with 20 μM final concentration of the TMPyP porphyrin. Complete inactivation of the model bacterium was successfully achieved, as verified by absence of luminescent CFUs on agar plates and a species-specific molecular technique that can detect the model organisms with high sensitivity (Multiplex PCR combined with Most Probable Number enrichment).

In chapter 5, possible toxicity of the cationic porphyrin and $^1\text{O}_2$ against two different types of *Artemia* cysts (magnetic and unmodified) was tested in dose-response experiments using percentage of cyst hatching as the measured variable. Surprisingly, magnetic cysts showed improved hatching under sub-optimal hatching conditions in the presence of porphyrin-generated ROS and the porphyrin TMPyP alone (i.e. independent to the production of $^1\text{O}_2$) relative to the controls. In contrast, dose-response experiments with unmodified cysts showed a positive hatching response only to TMPyP-generated $^1\text{O}_2$ but not TMPyP itself. Further investigations showed that when magnetic cysts were mixed with the model pathogen, complete pathogen inactivation was achieved after six hours of incubation with 20 μM of TMPyP in the light.

In conclusion, my research demonstrated that PACT is suitable as an additional (or alternative) sterilization method in prawn hatcheries and potentially for aquaculture water treatment in general.

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1 General Introduction: ¹Photosensitisers, Review of Their Application in Aquaculture

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1.1 Introduction

The Food and Agriculture Organisation of the United Nations (FAO) and Coordinating Working Party on Fishery Statistics (CWP) describe aquaculture as “the farming of aquatic organisms: fish, molluscs, crustaceans, aquatic plants, crocodiles, alligators, turtles, and amphibians” (FAO, 2013). Nowadays, different culture methods are used to obtain the best product from farmed animals. For instance, ponds (i.e. prawns, fishes, eels, crayfish), tanks (i.e. prawn brood stock tank, prawn culture tank), sea cages (i.e. salmon, tuna, snapper, mullet), long line (i.e. pearl oyster), raceways (i.e. abalone, oyster, algae) and hatcheries (where newborn organisms are grown) are the most common methods used worldwide (FAO, 2013).

Continued fishing and overfishing of natural resources has reached unsustainable levels, causing a decrease in natural fish stocks (FAO, 2012, 2016). Aquaculture is the fastest growing food production sector; in 2014, the aquaculture industry provided nearly 73.8 million tonnes (total value of US\$ 160.2 billion) of fish globally and 27.3 million tonnes of aquatic plants (total value of US\$ 5.6 billion) (FAO, 2016). Progress has been made in the last decades to reduce the environmental

¹ This chapter will be submitted as review to “Aquaculture” Journal.

footprint of the aquaculture industry. For instance, research showed that plant-based feed has potential for replacing fishmeal, reducing fisheries pressure on the environment (Chiu et al., 2016; Geurden et al., 2013). Currently, up to 80% fishmeal can be substituted, depending on plant-based feed and animal farmed (Kader and Koshio, 2012; Sarker et al., 2012). For example, one hundred percent of replacement of fish meal with terrestrial eco-friendly alternatives led to reduction of expression of several genes involved in myogenesis and muscle growth, weight, fish length and induced hepatocellular lesions (Rhodes et al., 2016). Therefore, aquaculture still largely depends on wild-caught fish for fishmeal and fishoil (Tacon et al., 2009).

There is room of improvement in aquaculture, not only in relation to feed quality, but also in regards to prophylactic measures for maintaining healthy animals and avoiding massive losses due to disease. As in other farming industries, infectious disease relating to pathogens, that live and proliferate at the expense of the host organism, is also an enormous problem in aquaculture (Saravanan et al., 2013). Different biological agents, such as bacteria, viruses, protists, helminths (worm-like polyphyletic group of eukaryotic pathogens) oomycete and fungi are responsible for animal diseases. Bacterial infections are responsible for heavy financial losses on aquaculture farms (Almeida et al., 2009; Austin and Austin, 2012; FAO, 2012; Saravanan et al., 2013), causing up to 22% of losses in the prawn farming sector (FAO 2014). Bacterial pathogens have been linked to food poisoning (Lozano-Leon et al., 2003) and mass mortality of larval and post larval stages of crustaceans (Cano-Gomez et al., 2010; Jithendran et al., 2010; Karunasagar et al., 1994; Payne et al., 2006; Saoud et al., 2013), fish (Austin, 2011; Romalde, 2002; Saravanan et al., 2013; Toranzo et al., 2005; Wang et al., 2016) and molluscs (Muroga, 2001; Tubiash et al., 1965) and were therefore selected as the main focus of the current study.

1.2 Bacteria Induced Diseases in Aquaculture

Like other organisms, bacteria can be divided into two groups based on their habitat source: indigenous and non-indigenous. While organisms belonging to the first group are part of the native flora of that particular environment (i.e. *Vibrio anguillarum*, *Vibrio vulnificus*, *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Photobacterium damsela* etc.), non-indigenous bacteria are introduced via

contamination, most commonly by animal excreta and human waste (i.e. *Salmonella* spp. and *Escherichia coli*) (Fukuda et al., 1996; Muroga et al., 1986; Muroga et al., 1987; Nakai and Park, 2002). During an outbreak, both indigenous and non-indigenous bacteria might cause either “acute disease”, which progresses quickly and generally results in high mortality, or “chronic disease”, which progresses slowly with a low mortality rate but lasts for a longer period (Austin and Austin, 2012). Pathogens are generally introduced to culture system and/or the host organism via vectorial transfer (Olafsen, 2001) (non-indigenous microorganisms) or they proliferate to levels beyond normal in the host because of high stress levels (mostly indigenous). When animal population density increases (i.e. intensive/super intensive aquaculture production), high-stocking density-induced stress leads to increased mucus secretion (Olafsen, 2001), compromised immune systems and decreased disease resistance of the animal farmed (Ndong et al., 2007; Rotllant et al., 1997). These stress symptoms are often linked to reduced water quality, which might create favourable conditions for a bacterial epidemic (Sung et al., 2011).

Among the different bacterial diseases in marine aquaculture (for a comprehensive list of fish diseases we suggest Austin and Austin (2012) and Saravanan et al. (2013)), Vibriosis and fish Pasteurellosis (Photobacteriosis) are important diseases in natural as well as in commercial production systems worldwide. These diseases can cause mass mortality events in aquaculture farms, with up to 100% mortality rates (Almeida et al., 2009; Karunasagar et al., 1994; Pizzutto and Hirst, 1995; Romalde, 2002; Toranzo et al., 1991; Zhang et al., 2014b). Vibriosis is associated with species of the closely related genera *Vibrio* and *Photobacterium*, including *Photobacterium damsela* subsp. *damsela*, *V. anguillarum*, *V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus*, *V. salmonicida*, *V. owensii*, *V. harveyi* etc. (Almeida et al., 2009; Austin and Austin, 2012; Saravanan et al., 2013). In contrast, Pasteurellosis is caused by *Photobacterium damsela* subsp. *piscicida*, formerly known as *Pasteurella piscicida* (Almeida et al., 2009; Austin and Austin, 2012; Saravanan et al., 2013; Toranzo et al., 1991).

Table 1.1: Common bacterial prawn diseases.

Name of disease	Bacterial species	Organs affected/ symptoms	References
Acute hepatopancreatic necrosis disease (AHPND)	<i>Vibrio parahaemolyticus</i>	Atrophied hepatopancreas (HP) and an empty stomach and midgut	(Nunan et al., 2014)
Bacterial necrosis	<i>Vibrio</i> spp, <i>Pseudomonas</i> spp, <i>Aeromonas</i> spp and <i>Spirillum</i> spp	Breakdown of chitin in the exoskeleton, leading to erosion and melanisation	(Jithendran et al., 2010)
Bolitas		Intestine and hepatopancreas	(FAO, 2007)
Epibiont fouling		Exoskeleton (head and body) and around the gills	(FAO, 2007)
Luminescent bacterial disease	<i>Vibrio harveyi</i> -clade bacteria	Luminescent coloration of animals in the dark	(Chrisolite et al., 2008; Karunasagar et al., 1994; Wang et al., 2015)
White patch disease (WPD)	<i>Bacillus cereus</i>	White opaque patches in the carapace, necrosis, whitish blue coloration, loss of appetite and pale white muscles	(Velmurugan et al., 2015)

Vibrio species are a widely distributed group of bacteria living in animal (and human) intestinal tracts (Thompson et al., 2004; Zanetti et al., 2001) and also occur in estuaries, marine coastal waters and sediments (Thompson et al., 2004). They are Gram-negative bacteria belonging to the Gammaproteobacteria (Thompson et al., 2004), characterised by curved rod shapes. They are usually motile (i.e. possess at least one flagellum), facultative anaerobes; meaning that they use aerobic respiration if oxygen is present but have the capacity to switch to anaerobic fermentation if necessary (Lozano-Leon et al., 2003; Thompson et al., 2004). Some of the most problematic illnesses in prawn farming are caused by *Vibrio* bacteria (Table 1.1), responsible for high mortality rates in larval stages as well as adults (Martin et al., 2004). The term ‘*Vibrio* disease’ includes diseases referred to as vibriosis, bacterial disease, penaeid bacterial septicaemia, penaeid vibriosis, luminescent vibriosis or red-leg disease (Aguirre-Guzmán et al., 2004; Wang et al., 2015).

Luminescent vibriosis (Table 1.1) is caused by *V. harveyi*-like bacteria and leads to catastrophic losses in prawn farms (Lio-Po et al., 2005). Bacteria belonging to the *harveyi*-clade, are capable of producing phenotypes controlled by a quorum sensing

system such as natural luminescence and the production of several virulence factors (Defoirdt et al., 2007a). The large strain-to-strain variation in pathogenicity and luminescence should be noted and there seems to be no link between the degree of luminescence and the virulence of a given strain (Defoirdt et al., 2008).

1.3 Biosecurity

Biosecurity is a recognised major concern for aquaculture industries and includes the practices, procedures and policies used to avoid the introduction and spread of pathogens (e.g., bacteria, viruses, fungi, other parasites) responsible for transmitting infections to farmed animals (Lee and O'Bryen, 2001).

Prevention and control of infections are not easily managed in aquaculture, due to the following pathogen characteristics: widespread, rapid growth (Almeida et al., 2009) and difficulties in disease outbreak prediction. Pathogens may be introduced with the use of wild brood stock, water, live food and by external predators (mainly birds in open ponds) (Department of Primary Industries and Fisheries, 2008; Shrimp News International, 2014). For example, microalgae and/or *Artemia* nauplii (brine shrimp) are commonly used globally as live feed and have been identified as microbial pathogen carriers and vectors (Defoirdt et al., 2006; Goulden et al., 2012; Han et al., 2007; Interaminense et al., 2014; Puente et al., 1992; Quiroz-Guzman et al., 2013). The use of routine biosecurity measures can reduce the risk of pathogen introduction, but, once introduced, effective pathogen control treatments need to be applied to limit infection of the farmed animals (Lee and O'Bryen, 2001). For instance, within the hatchery compartment of aquaculture farms, infections can start with bacteria colonising hatched eggshells or *via* live feed carriers. Indeed, *Vibrio* spp are associated with shell disease and midgut infections, but causes are not well understood and are attributed mainly to stress and environmental factors (Martin et al., 2004). When eggs hatch, opportunistic bacteria present in the system might use egg yolk as a carbon source, leading to exponential population growth (Martin et al., 2004; Quiroz-Guzman et al., 2013). Alternatively, as newly hatched larvae rely on egg yolk for initial nutrition (Sorgeloos et al., 1998), they inadvertently might ingest the colonising bacteria (Martin et al., 2004). Studies demonstrated that pathogenic *Vibrio* spp (*V. harveyi*-like bacteria) adhere to egg shells and, after prawn hatching, use the yolk reserve to proliferate

(Martin et al., 2004; Sung et al., 2001; Vandenberghe et al., 1999). Vectors such as live feed (microalgae or *Artemia*) have been identified as being important in the progression and the severity of the infection (Defoirdt et al., 2006; Interaminense et al., 2014; Martin et al., 2004; Puente et al., 1992). Interactions between algae and bacteria include commensalism, competition and parasitism (Natrah et al., 2014; Pintado et al., 2014; Salvesen et al., 2000; Unnithan et al., 2014). These interactions are regulated by production of metabolites such as organic exudates, toxins and other signalling molecules (Duff et al., 1966; Joint et al., 2002; Kogure et al., 1979). When the microalgae are moved from one environment (i.e. algae culture water) to another with different chemical and physical factors, the chemical composition of microalgae also changes (Reitan et al., 1994; Salvesen et al., 2000). Therefore, bacterial growth might (or might not) proliferate (Salvesen et al., 1999; Skjermo and Vadstein, 1993; Tubiash et al., 1965) and/or bacteria associated with microalgae cultures (Salvesen et al., 2000) could be ingested by farmed animals, especially when present in high concentrations.

Artemia spp. (brine shrimp) also seem to be an excellent carrier and vector for infections and perhaps the main route for vectorial transfer of pathogenic bacteria to crustacean and fish hatchery ponds, causing mass mortalities (Halet et al., 2007). Indeed, brine shrimp feed naturally on bacteria from development stage Instar II (8-12 h after hatching) (FAO, 2007; Sorgeloos et al., 1998), and vectorial transfer to the larvae occurs if they ingest brine shrimp with contaminated gut content (Goulden et al., 2012; Halet et al., 2007).

1.4 Choice of Study Organisms

1.4.1 Prawns

Prawns are crustaceans belong to the family Penaeidae. Of the 70 prawn species in Australia, ten are of commercial interest, with banana and tiger prawns being most commonly farmed in Queensland (Department of Agriculture Fisheries and Forestry, 2013). Mature prawns produce 50,000 to 1,000,000 eggs, with spawning normally occurring at night. Nauplii (first larval stage) hatch within 24 h and feed on their egg reserves for the first two days before metamorphosing into the second larval stage (zoea) (Shrimp News International, 2014). Zoea feed on algae for about 5 days before metamorphosing into mysis (final larval stage), which feed on both microalgae

and zooplankton (mainly *Artemia* nauplii) for almost four days before reaching the post-larval development stage.

1.4.2 Microalgae

Algal species such as *Tisochrysis lutea* (T-iso), *Nannochloropsis oculata*, *Tetraselmis chui*, *Picochlorum atomus* and *Chaetoceros muelleri*, are used worldwide as live feed for crustacean and fish larvae-culture (Brown and Blackburn, 2013; FAO, 2007; Salvesen et al., 2000). Their high protein -, amino acid -, carbohydrate - and polyunsaturated fatty acids (PUFA) content is of immense interest to the aquaculture industry. Macromolecular content in microalgae is highly variable, to a degree species-dependent (Finkel et al., 2016) and influenced by growth conditions (Guedes et al., 2010; Renaud et al., 2002; Thompson et al., 1992). When nutrients are not limited (standard growth conditions), macromolecule content can range from 25 and 50 % of dry weight for protein, between 5 and 40 % for carbohydrate, from 10 and 30 % for lipid and between 5 and 40 % for ash (Knuckey et al., 2002; Martinez-Fernandez et al., 2006; Renaud et al., 1999; Whyte, 1987). Microalgae have also other application such as high-energy food for human and animal consumption (Liu and Chen, 2016), biofuel (Borowitzka and Moheimani, 2010; Islam et al., 2013; Liu and Chen, 2016; Pandey et al., 2014; Sing et al., 2013), and for cosmetic applications (Fernandes et al., 2015).

1.4.3 Artemia

Artemia, known as brine shrimp, are small crustaceans belonging to the Artemiidae family. This small crustacean is capable to adapt to and survive in extremely unstable abiotic conditions (i.e. salinity, temperature, oxygen concentration, UV irradiation etc.) (Sorgeloos, 1980; Van Stappen, 1996, 2002). Survivor adaptation of this specimen to environmental stressors is evident in females via production of swimming larvae (nauplii) or encysted gastrulae (cysts) (MacRae, 2003). Cyst production aids to tolerate adverse environmental conditions, where the embryo is subjected to reversible physiological condition that cause suspension of development and a reduction in metabolism known as diapause (MacRae, 2003; MacRae, 2005). However, when cysts are hydrated, the embryo emerges from the diapause state

indicated by increased metabolism and ultimately excystment (Drinkwater and Clegg, 1991; Nambu et al., 2008).

Artemia is used in aquaculture worldwide due to the ability to produce cysts (Daintith, 1996). Indeed, cysts may be stored for long periods and hatched on demand to provide a convenient form of live feed for larval fish and crustaceans (Daintith, 1996). They are highly nutritive providing about 53% of protein, 20% lipids, 15% carbohydrates and 10% of ash to larvae and adults (Léger et al., 1987). Although hatching of *Artemia* cysts is relatively easy and simple (El-Magsodi et al., 2014; Sorgeloos et al., 2001), handling and growth conditions such as de-capsulation, disinfection, temperature, dissolved oxygen, pH and salinity can affect hatching rates, maximum performance and production cost (Lavens and Sorgeloos, 1996).

1.5 Traditional Methods to Prevent Disease in Aquaculture

1.5.1 Antibiotics and Vaccines

Diseases in aquaculture are a serious problem and different methods are used to prevent or to cure disease. Once farmed adult animals show signs of infection, antibiotics are often used (Bermúdez-Almada and Espinosa-Plascencia, 2012), however, to avoid mass mortalities in adult animals, vaccines are applied as a prophylactic measure. Despite the benefit of vaccines, they are limited to certain hatchery compartments as larvae have undeveloped immune systems (Bentzon-Tilia et al., 2016; Vadstein, 1997) and are almost too small and fragile for handling, especially on mass (Vadstein, 1997). On the other hand, while antibiotics are currently used (or overused) in adults and larvae (Bermúdez-Almada and Espinosa-Plascencia, 2012), their application in aquaculture should be restricted as they can induce bacterial resistance, induce human health problems (i.e. allergies) and accumulate in aquatic environments (Bermúdez-Almada and Espinosa-Plascencia, 2012; Cabello, 2006; Ma et al., 2006). The main concern is the potential for the development of antibiotic-resistance in bacterial pathogens, the likely transfer of resistance to land pathogens and modifications of bacterial flora in sediments and water (Cabello, 2006; Ma et al., 2006). *Vibrio* bacteria have the ability to attain resistance to antibiotics. Karunasagar et al. (1994) demonstrated that a *V. harveyi* antibiotic-resistant luminescent strain caused 100% prawn larvae mortality, and the microorganisms showed signs of luminescence

and a high concentration of bacteria in the haemocoel of larvae. Wang et al. (2015), also, observed that the antibiotic-resistant strains, CAIM 333 and CAIM 372, of *V. campbellii* were pathogenic to the zoea stage of *Litopenaeus vannamei*.

Hence, larvae are more vulnerable to microbial infections and the development of good husbandry practices and alternative microbial management methods are essential for successful hatchery operations.

1.5.2 Water Treatment

Aquaculture water has been identified as the main carrier of potentially infectious microbial pathogens (Fernandes et al., 2010; Kim and Lee, 2017). Ultra violet (UV-C) radiation (direct photolysis), ozone (O₃) and chlorine (Cl₂) are commonly used treatments for sterilising aquaculture water (Acher et al., 1997). The advantage of direct photolysis is that the high energy of the UV-C wavelengths is used for disinfection and no other chemical additives are required. On the other hand, the light-quenching properties of organic substances, can significantly reduce the efficiency of direct photolysis (inner filter effect) and this can be a significant problem for aquaculture water which often have a high organic load. In addition, direct photolysis technologies are expensive (resource- and energy-demanding: e.g. electricity, short life time of UV-C lamps and quartz sleeves) due to the large volumes of recirculated water within aquaculture ponds. Sterilisation of seawater using ozone and chlorine can have a positive effect on animal health (Wietz et al., 2009), however due to sensitivity of larvae to toxic by-products, uncontrolled applications can cause deformities and death to farmed animals (Hall et al., 2013; Jensen et al., 2011). Additionally, energy-intensive (ozone) and resource-intensive (ozone and chlorine), are discouraged in a fossil-fuel-constrained economy (Royal Society of Chemistry, 2007).

As a consequence, novel environmentally friendly water treatment technologies are required to make aquaculture more sustainable in a fossil-fuel constrained economy. Indeed, using effective and low cost technologies would help to reduce industry costs, consequently, the final price of the product. Alternative water treatment technologies that have been investigated for use in aquaculture are

described in Table 1.2 and grouped based on whether they target specific pathogenic bacteria (“pathogen-specific”) or not (“non-specific”).

Details of other novel sterilisation techniques will not be covered in this review as photodynamic antimicrobial chemotherapy (PACT) was the main focus of the current thesis and Table 1.2 below reports a comparison of alternative water treatment technologies.

Table 1.2: Selected novel water treatment technologies.

Technology	Type of action on organisms	Reference
Addition of short-chain fatty acids	Non-specific	(Defoirdt et al., 2006; Shivu et al., 2007)
Microalgae	Non-specific	(Ali et al., 2016; Defoirdt et al., 2010; Defoirdt et al., 2006; Lio-Po et al., 2005)
Quorum-sensing disruption	Pathogen-specific	(Defoirdt et al., 2010; Halet et al., 2007; Zhang and Li, 2016)
Phage therapy	Pathogen-specific	(Almeida et al., 2009; Busico-Salcedo and Owens, 2013; Oliveira et al., 2012; Pereira et al., 2017)
Photodynamic Antimicrobial Chemotherapy	Non-specific	(Alves et al., 2015b; Kanyal et al., 2016)
Polyhydroxyalkanoates	Non-specific	(Halet et al., 2007; Laranja et al., 2014; Lio-Po et al., 2005)
Probiotics	Non-specific	(Lakshmi et al., 2013; Newaj-Fyzul et al., 2014; Pintado et al., 2014; Prado et al., 2010)

1.6 Photodynamic Antimicrobial Chemotherapy (PACT)

1.6.1 What is PACT?

In the last decade, research has shifted attention to new technologies able to replace (or add to) the more traditional water treatment in aquaculture. Photodynamic Antimicrobial ChemoTherapy (PACT) showed a great potential as a non-specific method to kill microorganisms without harming the farmed animal (Alves et al., 2011a; Carey, 1992; Jori and Brown, 2004; Magaraggia et al., 2006).

PACT is achieved by photoexcitation of an organic dye (photosensitiser (PS)) producing reactive oxygen species (ROS) and radicals such as singlet oxygen ($^1\text{O}_2$), hydrogen peroxide (H_2O_2), superoxide ($\text{O}_2^{\bullet-}$) and hydroxyl radical ($\text{HO}\bullet$) in a series of photophysical reactions (Alves et al., 2015b; Jori and Coppellotti, 2007; Kanyal et al., 2016; Stojiljkovic et al., 2001). Molecular oxygen exists as a biradical when it is in a ground state (O_2) but, due to its spin-multiplicity, it is referred to as triplet oxygen

($^3\text{O}_2$). Absorption of light by the PS generates an excited singlet state ($^1[\text{PS}]^*$), which rapidly loses energy, returning to singlet ground state with fluorescence production or undergoes in intersystem crossing (ISC, Figure 1.1a) to its corresponding long-lived triplet excited state ($^3[\text{PS}]^*$, Figure 1.1a) (Alves et al., 2015b; Benov, 2015; Denis et al., 2011; Ochsner, 1997; Stojiljkovic et al., 2001). The triplet excited state can produce phosphorescence and returning to ground state (Alves et al., 2015b; Benov, 2015; Denis et al., 2011; Sperandio et al., 2013; Yoon et al., 2013). Another possibility is that electrons can be transferred from or to a substrate. Electron transfer from $^3[\text{PS}]^*$ to $^3\text{O}_2$ produces $\text{O}_2^{\cdot-}$ (Type I pathway), which is a radical intermediate and can yield formation of other ROS such as H_2O_2 , and HO^{\cdot} (Alves et al., 2015b; Benov, 2015; Denis et al., 2011; Ochsner, 1997; Sperandio et al., 2013; Stojiljkovic et al., 2001; Yoon et al., 2013). Alternatively, the energy can be transferred from $^3[\text{PS}]^*$ through collision with $^3\text{O}_2$, which induces structural modifications (i.e. change in spin orientation in O_2) and leads to the formation of the higher-energy $^1\text{O}_2$ state (Type II pathway), returning the PS to its ground state (Figure 1.1a and b) (Alves et al., 2015b; Benov, 2015; Denis et al., 2011; Jori and Coppelotti, 2007; Ochsner, 1997; Sperandio et al., 2013; Stojiljkovic et al., 2001; Yoon et al., 2013). Although both pathways (type I and II) occur simultaneously, the Type II reaction and formation of $^1\text{O}_2$ is widely considered as primarily responsible for the photoinactivation of organisms using porphyrins as PS but depends on the experimental conditions (Foote, 1991; Hadjur et al., 1998; Maclean et al., 2008; Maisch et al., 2005; Muller-Breitkreutz et al., 1995; Nitzan et al., 1989; Ochsner, 1997). Singlet oxygen, depending on its concentration, is also toxic to most cells (i.e. photoinactivation) (Alves et al., 2011a; Dahl et al., 1987; Magaraggia et al., 2006; Snyder et al., 2006) and therefore information presented here take in consideration mainly the type II pathway.

1.6.2 Photosensitisers

Generally included in the term photosensitiser (PS) are molecules that are activated (“excited”) when exposed to a light source and able to interact with O_2 and producing ROS. The principal characteristic of PS is that its absorption spectrum has to coincide with the wavelength of the chosen light source. It also should show a high efficiency in producing $^1\text{O}_2$ (DeRosa and Crutchley, 2002; Jori and Brown, 2004), which

is expressed in the quantum yield ($\Phi\text{-}^1\text{O}_2$). Many classes of photosensitisers have been utilised for $^1\text{O}_2$ disinfection, in particular organic dyes (rose bengal, eosin, and methylene blue), synthetic dyes such as fullerenes (Mroz et al., 2007) and natural-derivate dyes (porphyrins, phthalocyanines and related tetrapyrrolic macrocycles) (Almeida et al., 2009). Among the various known PS-dyes, porphyrins have been efficiently used for disinfecting drinking water (Bonnett et al., 2006), waste water (Carvalho et al., 2009; Jemli et al., 2002) and aquaculture water (Alves et al., 2011a; Magaraggia et al., 2006).

Porphyrins are heterocyclic molecules composed of tetrapyrrole rings joined by a methine bridge (=CH). Porphyrins can be of natural origin or be prepared in the laboratory and, depending on the overall charge, they can be cationic, anionic or neutral. The properties of these dyes depend on the different molecular structure and presence or absence of a metal ion in the centre of the tetrapyrrole ring structure, affecting absorption spectra and spectral ranges (from 390 to 650 nm) (Pavinatto et al., 2008). The best known class of tetrapyrroles produced naturally by plants and algae are the chlorophylls (Wohllebe et al., 2012). Natural porphyrins play important roles in photosynthesis (e.g. chlorophylls), oxygen transport (e.g. haemoglobin) and redox-reactions (e.g. cytochrome P450) (Almeida et al., 2009). Most porphyrins themselves, at micromolar (μM) concentration, are non-toxic to fish (Almeida et al., 2011; Magaraggia et al., 2006) and to humans (Almeida et al., 2011; Maisch, 2009; O'Connor et al., 2009; Ortner, 2009; Smith et al., 2009) and, as a result, are found in foods (European Union, 1989). Unlike antibiotics, porphyrins are improbable to accumulate in aquatic environments due to photobleaching when exposed to irradiation (Bonnett and Martínez, 2001; Kuznetsova et al., 2010; Rotomskis et al., 1997).

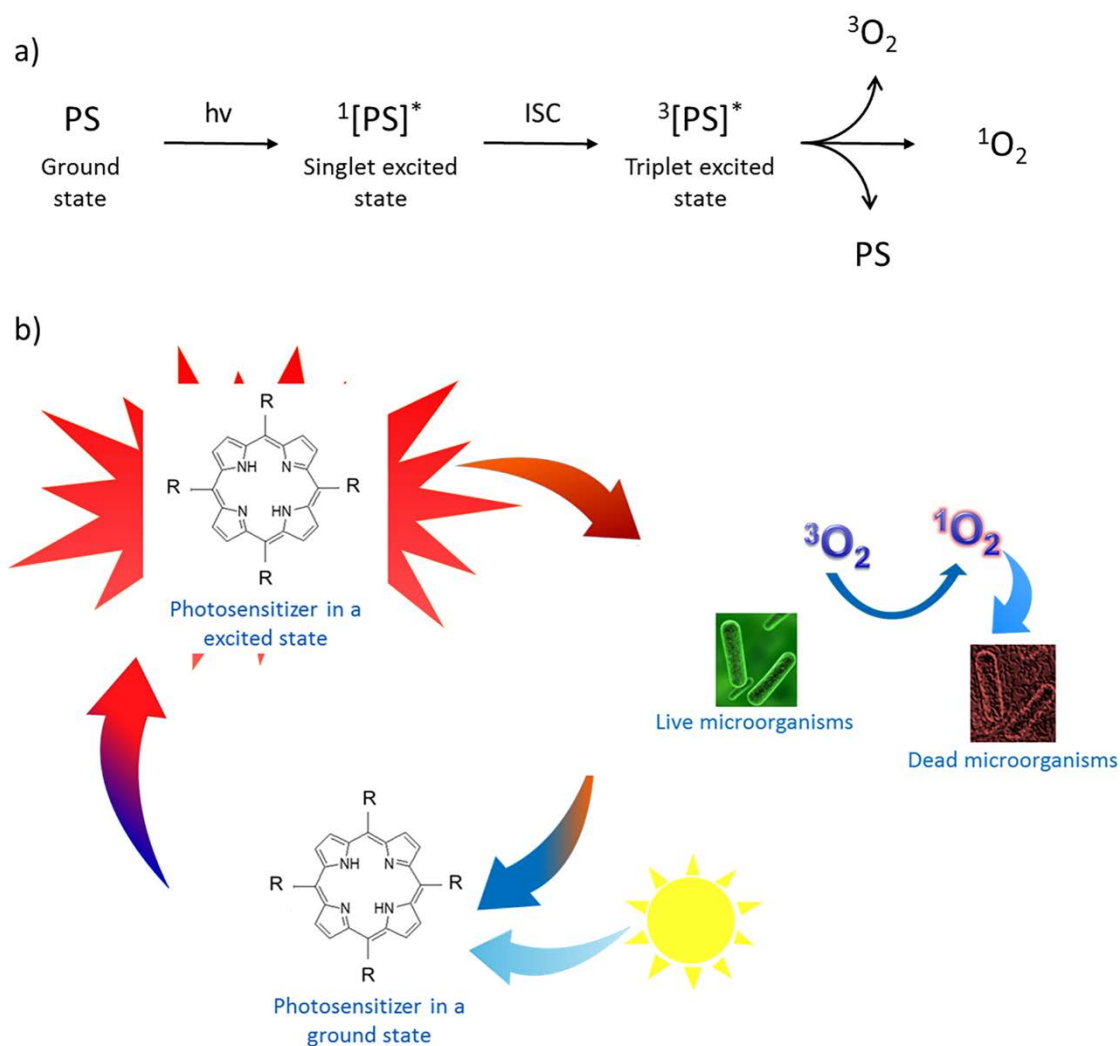


Figure 1.1: Graphic representation of PS excitation and singlet oxygen (${}^1\text{O}_2$) production during PACT. a) equation of singlet oxygen (${}^1\text{O}_2$) production, PS = photosensitizer in ground state, ${}^1[\text{PS}]^*$ = photosensitizer in a singlet excited state, ${}^3[\text{PS}]^*$ = photosensitizer in a triplet excited state, ${}^3\text{O}_2$ = triplet oxygen and ${}^1\text{O}_2$ = singlet oxygen; b) Schematic illustration of singlet oxygen (${}^1\text{O}_2$) production using porphyrins when the PS is irradiated by visible light.

Not many studies have been conducted using porphyrins in aquaculture; however, isolated examples of aquaculture applications have been reported (Figure 1.2). For instance, ${}^1\text{O}_2$ disinfection of pond water showed outstanding performance for preventing fish infections (Jemli et al., 2002). *Saprolegnia* infection (a pathogenic fungus) in rainbow trout was also cured using cationic porphyrins and low intensity visible light irradiation (Magaraggia et al., 2006). Alves et al. (2011b) demonstrated that the natural luminescent *Vibrio fisheri* was photoinactivated using cationic porphyrins in aquaculture water and how the seasonal difference in aquaculture water quality affected the efficiency of the method. Malara et al. (2017b) completely inactivated *V. owensii* and *V. parahaemolyticus*, known pathogens for lucrative

aquaculture organisms (Aguirre-Guzman et al., 2010; Cano-Gomez et al., 2010), using both a cationic and a neutral porphyrin after 24 h of continuous irradiation of the bacteria in the culture medium. Therefore, $^1\text{O}_2$ may be a promising approach to control *Vibrio* pathogen loads in aquaculture water on industrial-scales (Maisch, 2007).

1.6.3 Mechanism of Photosensitized Singlet Oxygen Production

The $^1\text{O}_2$ has a lifetime of about 3-4 μs in aqueous - (Ochsner, 1997; Rodgers and Snowden, 1982) and $\sim 10 \mu\text{s}$ in lipid environments (Jori and Coppellotti, 2007; Ochsner, 1997), which is long compared to other reactive oxygen species such as $\text{HO}\bullet$ (<10 ns) (Roots and Okada, 1975), allowing it to diffuse over relatively longer distances (Foote, 1991; Ochsner, 1997). Singlet oxygen can penetrate through cell membranes, although this process is less efficient (Skovsen et al., 2005). More importantly, the PS actively interacts with cell walls and cell membranes and, upon irradiation, produces $^1\text{O}_2$ in proximity of the cell wall or membrane.

Generally, the outer wall and cytoplasmic membrane structure of Gram-positive and Gram-negative bacteria play a crucial role in protecting the cell. Based on their charge, PS are capable of cell adherence inducing cellular photooxidation (Zeina et al., 2001). The peptidoglycan cell wall of Gram-negative bacteria is covered by a highly organised and negatively charged outer membrane composed of phospholipids, lipopolysaccharides, polysaccharides, proteins and lipoproteins (Maisch et al., 2004), making it impermeable to molecules larger than 700 Da (Jori and Coppellotti, 2007; Jori et al., 2006). Commonly used PS during PACT application have molecular weights not exceeding 1,800 Da (Jori et al., 2006) thus, cationic porphyrins might interact first with negatively charged compounds in the outer membrane of Gram-negative bacteria (electrostatic attraction) and could also be taken up by a postulated self-promoting uptake pathway despite their heavy weight (Jori and Coppellotti, 2007). In contrast, free transport across membranes is discouraged for negatively charged porphyrins that are electrostatically repelled (Benov, 2015; Jori and Coppellotti, 2007) and neutral porphyrins being nonpolar might (or might not) penetrate the negatively charged lipopolysaccharide outer membrane (Jori and Coppellotti, 2007). Anionic PS, however, might be able to diffuse across membrane when the number of charges is two as lipophilicity increases (Benov, 2015; Castano et al., 2004). Higher numbers of negative

charges (more than two) impede active transport of anionic porphyrins (Boyle and Dolphin, 1996; Castano et al., 2004). In contrast to Gram-negative bacteria, molecules of molecular weight up to 60,000 Da are able to diffuse through the porous peptidoglycan layer containing bridging lipoteichoic and teichuronic acids in the outer membrane of Gram-positive bacteria (Friedrich et al., 2000; Jori and Coppellotti, 2007; Jori et al., 2006). Therefore, it is generally accepted that cationic compounds are more efficient against Gram-negative and -positive bacteria, while anionic and neutral ones are effective mainly against Gram-positive microorganisms (Hamblin and Hasan, 2004; Maisch et al., 2004; Malik et al., 1990; Merchat et al., 1996a; Merchat et al., 1996b; Nitzan et al., 1992). Merchat et al. (1996a) showed that a tetra-cationic porphyrin was more efficient than the anionic counterpart against *Enterococcus seriolicida*, (Gram-positive), and *Vibrio anguillarum*, (Gram-negative). In contrast, isolated studies showed efficient anti-bacterial effects of a neutral (Malara et al., 2017b) and a cationic porphyrin against Gram-negative bacteria, leading to the conclusion that light, exposure time, nature of the aqueous media and/or cell concentration might play an important role in the efficiency of the selected compound (Alves et al., 2015b; Coppellotti et al., 2012; Jori et al., 2011; Vatansever et al., 2013).

1.6.4 Singlet Oxygen Cellular Damage

The efficiency of PACT during the photosensitization depends on the physical PS localization and the abundance of target biomolecules (Bacellar et al., 2015; Vatansever et al., 2013). Singlet oxygen in proximity to biological macromolecules can cause hydroperoxides to react with unsaturated double bonded carbon-carbon or endoperoxides to react with conjugated diene systems (Glaeser et al., 2011; Ryter and Tyrrell, 1998). Singlet oxygen is also able to react with alkenes (containing sulfur- or nitrogen), phenol or sulphides producing 1,2-dioxetanes, hydroperoxydienones and sulfoxides, respectively (Glaeser et al., 2011; Ryter and Tyrrell, 1998).

Table 1.3 summarises major cellular targets of singlet oxygen with proteins (amino acid such as histidine, tryptophan, tyrosine, cysteine, and methionine) being one of the principal target due to cellular abundance (Alves et al., 2015a; Benov, 2015; Davies, 2004; Glaeser et al., 2011). Protein oxidation leads to a decrease in amino acid

abundance and subsequent accumulation of toxic, un-stable substances (peroxides) or stable products such as kynurenine or sulfoxides (Davies, 2003).

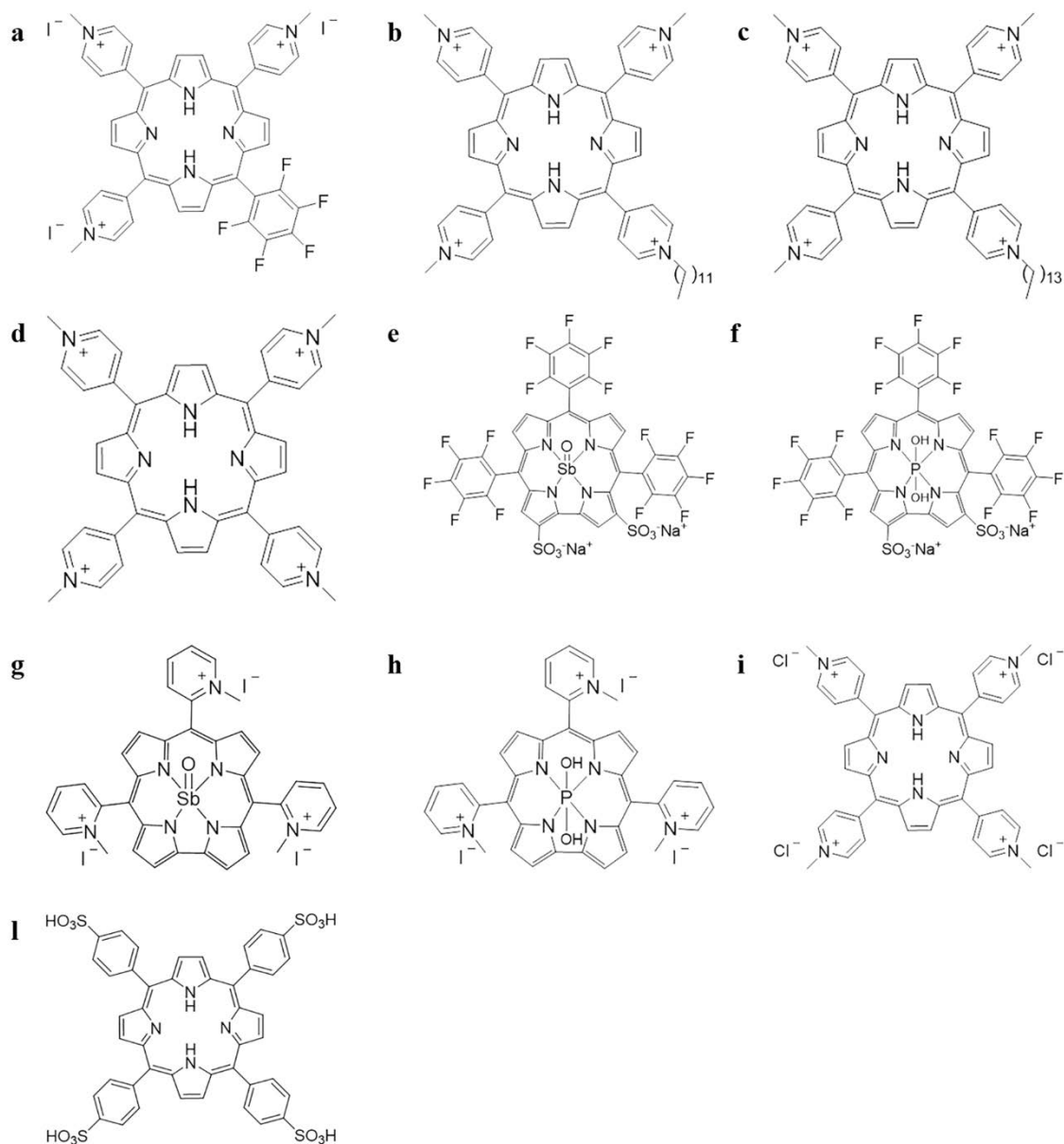


Figure 1.2: Example of PS used in aquaculture related studies. ^a 5,10,15-tris (1-methylpyridinium-4-yl)-20-(pentafluorophenyl) porphyrin tri-iodide (Alves et al., 2011b; Arrojado et al., 2011); ^b meso-tri (N-methyl-pyridyl) mono (Ndodecyl-pyrydyl) porphine (C12) (Fabris et al., 2012; Schrader et al., 2010); ^c Tri-meso (N-methyl-pyridyl), meso (N-tetradecyl-pyridyl) porphine (C14) (Magaraggia et al., 2006); ^d Tetra-meso (N-methyl-pyridyl) porphine (C1) (Magaraggia et al., 2006); ^e 2,17-bis-sulfonato-5,10,15-tris (pentafluorophenyl) corrolato (oxo) antimony(V) (Pohl et al., 2015); ^f 2,17-bis-sulfonato-5,10,15-tris (pentafluorophenyl) corrolato (trans-dihydroxo) phosphorus(V) (Pohl et al., 2015); ^g 5,10,15-tris-(N-methyl-o-pyridylium) corrolato (oxo) antimony(V) (Pohl et al., 2015); ^h 5,10,15-tris-(N-methyl-o-pyridylium) corrolato (trans-dihydroxo) phosphorus(V) (Pohl et al., 2015); ⁱ 5,10,15,20-tetrakis [N-methyl-4-pyridyl] porphyrin (Malara et al., 2017b); ^j 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrin (Malara et al., 2017b).

Another main target of $^1\text{O}_2$ are the membrane lipids especially unsaturated fatty acid (Girotti, 2001; Girotti and Kriska, 2004). Oxygen has a high solubility in lipid environments, providing more opportunity for PS localized in vicinity to lipid membrane to encounter oxygen and generate $^1\text{O}_2$ compared to the aqueous environment (Benov, 2015). When $^1\text{O}_2$ is generated, the reaction with unsaturated fatty acids produce lipid peroxides (Girotti and Kriska, 2004) causing a destabilization of the cell membrane and eventually death of the targeted microorganisms.

Table 1.3: Effect of $^1\text{O}_2$ on cell biomolecules.

Target Bio-macromolecules	$^1\text{O}_2$ effect	Reference
Amino acids	Histidine (oxygen addition), Tryptophan (oxygen addition) Cysteine (disulfide) Methionin (sulfoxide)	(Davies, 2003)
Cholesterol	7-hydroperoxides	(Girotti, 2001; Girotti and Kriska, 2004)
Lipids	Lipid hydroperoxides	(Girotti, 2001; Girotti and Kriska, 2004)
Nucleic acids	8-oxo-7,8-dihydroguanosine	(Cadet et al., 2010; Cadet et al., 2006)
Proteins	Cross-linking, Proteolysis	(Davies, 2003, 2004)

While nucleic acids are not the main target of $^1\text{O}_2$ (Alves et al., 2013; Awad et al., 2016; Bertoloni et al., 1992; Nitzan and Ashkenazi, 2001; Schafer, 1998), oxidation of DNA results in genotoxicity and mutagenicity (Castano et al., 2005) and can lead to cell death (Benov, 2015). Singlet oxygen with nucleic acids is highly selective (Epe, 1991), showing preference for guanine bases (Cadet et al., 2010) oxidising guanine to 8-oxo-7,8-dihydro-20-deoxyguanosine (Cadet et al., 2010; Cadet et al., 2006).

1.6.5 PACT Application

PACT was accidentally discovered at the beginning of 1900 when, for the first time, acridine hypochlorite dye in combination with visible light showed a lethal effect on the eukaryotic protist *Paramecium infusoria* (Raab, 1900). Following his student experiments, Professor Von Tappeiner (1904, 1909) used fluorescent dyes and light to inactivate the bacterium *Proteus vulgaris*. Von Tappeiner, named this process for the first time as “photodynamic action” (Von Tappeiner, 1904, 1909). Other studies by Von

Tappeiner were conducted to treat cutaneous diseases (i.e. condylomata lata, lupus vulgaris, psoriasis, stage II syphilis, and non-melanoma skin cancer) using eosin red solution as a PS and light (Szeimies et al., 2001). However, interest in PACT application in medical and clinic studies increased only in the second half of 1900 (Table 1.4) due to the discovery of the hematoporphyrin derivative (HpD) successively commercialized as Photofrin (Yoon et al., 2013). Only recently, less than 30 years ago, PACT was clinically approved as a non-invasive, highly selective method for killing undesirable cells and tissues and referred to as photodynamic therapy (PDT). For instance, in oncology, PDT was successfully used to treat selected tumours (Dougherty et al., 1998; Mitton and Ackroyd, 2008). Since then, PDT has been applied in other medical areas such as cardiology, immunology, ophthalmology, urology, dentistry and dermatology, as well as in the cosmetics industry (Babilas et al., 2010; Bozzini et al., 2012; Cotter, 2009; Konopka and Goslinski, 2007; Lee and Baron, 2011; Meisel and Kocher, 2005; Michels and Schmidt-Erfurth, 2001; Silva et al., 2008; Szeimies et al., 2013; Taub, 2012; Waksman et al., 2008; Wan and Lin, 2014; Woodburn et al., 1996). PACT was also tested against pathogens in blood products and for disinfection and sanitation of surfaces (Berg et al., 2007; Brovko, 2010; Maisch et al., 2012; Marciel et al., 2017; Wainwright, 2002). The interest in using PACT to cure microbial infections and lesions started only at a later stage, coinciding with the end of “antibiotic Era”. The idea to use an innovative method that would be non-toxic to mammalian cells and could kill microorganisms in quickly without creating resistant strains encouraged scientists to investigate possible PACT applications (Hamblin and Hasan, 2004; Jori et al., 2006). As described in Table 1.4, PACT was not only tested against antibiotic-resistant bacterial but also viral and fungal infections (Arenas et al., 2013; Calzavara-Pinton et al., 2012; Carre et al., 1999; Cormick et al., 2009; Costa et al., 2012; Felgentrager et al., 2013; Paz-Cristobal et al., 2014; Pereira Gonzales and Maisch, 2012; Sperandio et al., 2013; Wainwright, 2003). The transition from medical to veterinary applications was quick (Table 1.4) and focussed primarily on curing cancer, as the PS accumulates near the tumour cells and, once activated by the light, destroys the cancer cells (Buchholz and Walt, 2013). Despite the oncological application, dermatologic diseases were also successfully *in vitro* (Wardlaw et al., 2012) and *in vivo* as demonstrated recently by

applying PACT to cure pododermatitis in penguins (Nascimento et al., 2015; Sellera et al., 2014).

Table 1.4: Examples of studies in medical, veterinary, and environmental applications using PDT or PACT.

Field of application	Area of application	References
Medical/ clinical	Oncology	(Dougherty et al., 1998; Mitton and Ackroyd, 2008)
	Cardiology	(Waksman et al., 2008; Woodburn et al., 1996)
	Urology	(Bozzini et al., 2012)
	Immunology	(Cotter, 2009)
	Ophthalmology	(Michels and Schmidt-Erfurth, 2001; Silva et al., 2008)
	Dentistry	(Konopka and Goslinski, 2007; Meisel and Kocher, 2005)
	Dermatology	(Babilas et al., 2010; Lee and Baron, 2011; Wan and Lin, 2014)
	Cosmetics	(Szeimies et al., 2013; Taub, 2012)
	Surfaces sanitation	(Berg et al., 2007; Brovko, 2010; Maisch et al., 2012)
	Blood disease	(Wainwright, 2002)
	Viral disease	(Costa et al., 2012; Wainwright, 2003)
	Bacterial disease	(Arenas et al., 2013; Sperandio et al., 2013)
Fungal disease	(Calzavara-Pinton et al., 2012; Carre et al., 1999; Cormick et al., 2009; Felgentrager et al., 2013; Paz-Cristobal et al., 2014; Pereira Gonzales and Maisch, 2012)	
Veterinary	Oncology	(Buchholz and Walt, 2013; Wardlaw et al., 2012)
	Pododermatitis in penguins	(Nascimento et al., 2015; Sellera et al., 2014)
Environmental	Anti-parasite	(De Souza et al., 2014; Dondji et al., 2005; El-Tayeb et al., 2013; Erzinger et al., 2011; Kessel and Smith, 1989; Lucantoni et al., 2011; Robinson, 1983)
	Anti-viral	(Costa et al., 2012; Floyd et al., 2004)
	Anti-fungal	(Calzavara-Pinton et al., 2012; Carre et al., 1999; Cormick et al., 2009)
	Anti-bacterial	(Alves et al., 2009; Alves et al., 2011a; Alves et al., 2014; Demidova and Hamblin, 2004; Egles et al., 2010; Grinholc et al., 2015; Harris and Pierpoint, 2012; Jori and Brown, 2004; Komerik et al., 2000; Mesquita et al., 2014; Sperandio et al., 2013; Valkov et al., 2014)
	Algaecidal	(Drabkova et al., 2007; Jancula et al., 2008; McCullagh and Robertson, 2006a; McCullagh and Robertson, 2006b, c; Pohl and Röder, 2015; Pohl et al., 2015; Schrader et al., 2010)
	Aquaculture treatment	(Bonnett et al., 2006; Carvalho et al., 2009; Coppellotti et al., 2012; Jori et al., 2011; Likodimos et al., 2010; Magaraggia et al., 2006; Pansonato et al., 2011)
Aquaculture	Wastewater treatment	(Alves et al., 2008; Alves et al., 2009; Alves et al., 2011b; Arrojado et al., 2011; Costa et al., 2008; Tavares et al., 2011)
	Fish infections	(Magaraggia et al., 2006; Wohllebe et al., 2012)
	Prawn infections	(Suzuki et al., 2000)
	Feed disinfection	(Asok et al., 2012; Fabris et al., 2012; Pellosi et al., 2013; Peloi et al., 2008)

The discover of photobleaching properties of dye-based PS in the middle of 1980s (Moan, 1986) opened new fields of applications, such as in the environment and in aquaculture. The possibility to use a substance that after application disappears from the solution was the main reason to apply PACT to sterilize or disinfect water including drinking water and wastewater (Bonnett et al., 2006; Carvalho et al., 2009; Coppellotti et al., 2012; Jori et al., 2011; Likodimos et al., 2010; Magaraggia et al., 2006; Pansonato et al., 2011). Environmental studies also investigated the killing effect on virus, bacteria and fungi that were not of medical concern (Alves et al., 2009; Alves et al., 2011a; Alves et al., 2014; Calzavara-Pinton et al., 2012; Carre et al., 1999; Cormick et al., 2009; Costa et al., 2012; Demidova and Hamblin, 2004; Egles et al., 2010; Floyd et al., 2004; Grinholc et al., 2015; Harris and Pierpoint, 2012; Jori and Brown, 2004; Komerik et al., 2000; Mesquita et al., 2014; Sperandio et al., 2013; Valkov et al., 2014), but the innovative and interesting PACT applications are those against parasites and algae (De Souza et al., 2014; Dondji et al., 2005; Drabkova et al., 2007; El-Tayeb et al., 2013; Erzinger et al., 2011; Jancula et al., 2008; Kessel and Smith, 1989; Lucantoni et al., 2011; McCullagh and Robertson, 2006a; McCullagh and Robertson, 2006b, c; Pohl and Röder, 2015; Pohl et al., 2015; Robinson, 1983; Schrader et al., 2010). PACT was only investigated in aquaculture in the last 15 years (Table 1.4), showing interesting results to cure infection in farmed animals (Magaraggia et al., 2006; Suzuki et al., 2000; Wohllebe et al., 2012), disinfect feed (Asok et al., 2012; Fabris et al., 2012; Pellosi et al., 2013; Peloi et al., 2008) and for water treatment (Alves et al., 2008; Alves et al., 2009; Alves et al., 2011b; Arrojado et al., 2011; Costa et al., 2008; Tavares et al., 2011).

1.7 Aims and Objectives

The principal aim of the present work was to investigate the possible applicability of PACT to aquaculture hatchery farming of *P. monodon*. To the best of my knowledge, application of PACT related to hatcheries of prawn farms and specifically to *harveyi*-like bacteria as model organism have never been investigated. To achieve this principal aim, experiments were designed to investigate 1) the potential sterilization of hatchery water seeded with the chosen model bacterium (chapters 2 and 3) and 2) the sensitivity of and sterilization potential of the cationic

porphyrin for live feed organisms (microalgae and *Artemia*) in mixed culture with the model bacterium (chapter 4 and 5).

In the method development chapter (chapter 2), the aim was to select the model bacterium to be used in the following chapters based on the potential pathogenicity towards *P. monodon* after injection and luminescence intensity.

In chapter 3, TMPyP and TPPS₄ porphyrins were used to 1) confirm their suitability for aquaculture purposes comparing their photostability in seawater and 2) investigate their capacity to inactivate and kill the model bacterium.

In chapter 4, porphyrin toxicity towards microalgae during a six-hour dose-response treatment with TMPyP were first evaluated and only PACT-resilient microalgae were tested in mixed culture with the model bacterium. Viability of microalgae cells was evaluated using flow cytometry based on chlorophyll auto fluorescence and the live-dead stain Propidium Iodide (PI). The treatment time was chosen based on bacterial disinfection results shown obtained in the previous chapter (chapter 3). The PACT-resilient microalga, *N. oculata* was selected and used in mixed culture with *V. campbellii* ISO7 to investigate sterilization success in a contaminated feed algal culture.

In chapter 5, two different *Artemia* cysts (magnetic and unmodified) were tested for possible toxicity of the cationic porphyrin. Additionally, investigations explored antimicrobial efficiency of TMPyP in experiments where magnetic cysts were mixed with the model bacterium.

2 Chapter 2: ² Selection of Model Organism *via* Vibrio Challenge

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2.1 Introduction

Some of the most pathogenic bacteria for aquatic vertebrates and invertebrates belong to the Harveyi clade (Busico-Salcedo and Owens, 2013; Darshanee Ruwandeeepika et al., 2012; Nunan et al., 2014; Vanmaele et al., 2015). Species comprising the Harveyi clade include *V. alginolyticus*, *V. azureus*, *V. campbellii*, *V. harveyi*, *V. jasicida*, *V. mytili*, *V. natriegens*, *V. owensii*, *V. parahaemolyticus*, *V. rotiferianus*, *V. sagamiensis* (Urbanczyk et al., 2013). Within the Harveyi clade, species closely related to *Vibrio harveyi* include strains that express luminescence and cause luminescent vibriosis disease in crustacean (mainly prawn) hatcheries (Busico-Salcedo and Owens, 2013).

The virulence mechanisms of species closely related to *Vibrio harveyi* are poorly understood partly due to multiple virulence factors, high genome plasticity, multiple host species, and previous misidentification of strains (Austin and Zhang, 2006; Cano-Gomez et al., 2009; Cano-Gomez et al., 2011; Owens and Busico-Salcedo, 2006). Bacterial pathogenicity is highly strain-dependent and strains of the same species may have different host ranges (Austin and Zhang, 2006).

² Experiment with *V. campbellii* ISO7 published in: Malara, D., Høj, L., Heimann, K., Citarrella, G., Oelgemöller, M., 2017a. Capacity of cationic and anionic porphyrins to inactivate the potential aquaculture pathogen *Vibrio campbellii*. *Aquaculture*. 473, 228-236.

To establish if a microorganism is a potential virulent strain towards a specific host, experimental challenge experiments using the pure culture can be conducted. In challenge experiments, infections are often induced *via* injection, immersion or oral administration using feed or vectors (Saulnier et al., 2000). Scientists rely on Koch's postulate to confirm the pathogenicity of a specific strain. The original postulates included three important points that has been translated by Rivers (1937): "first that the parasite occurs in every case of the disease in question, and under circumstances which can account for the pathological changes and clinical course of the disease; secondly, that it occurs in no other disease as a fortuitous and non-pathogenic parasite; and thirdly, that it, after being fully isolated from the body and repeatedly grown in pure culture, can induce the disease anew; then the occurrence of the parasite in the disease can no longer be accidental, but in this case no other relation between it and the disease except that the parasite is the cause of the disease can be considered". Only in later stages was a fourth postulate introduced requiring that the microorganism needed to be re-isolated from a "clinically inoculated host" (Fredricks and Relman, 1996). According to Fredricks and Relman (1996), Koch's postulates present some limitations especially if considering viral disease but also clinical diseases in general. Even Koch was not able to fulfil the postulate with some infectious agents such as *Vibrio cholerae*, failing to validate the third postulate (Evans, 1976). Nowadays, using molecular techniques and better knowledge on virulence mechanisms, we are able to precisely identify microorganism associated with a diseased animal and to understand possible failing causes.

In this chapter, I aim to find a suitable model bacterial strain that is virulent to *P. monodon*, using virulence and bioluminescence as key factors that can be used in the subsequent chapters. An ideal model bacterium would be luminescent, so that it can be used in rapid luminescence-based assays, and is virulent to prawns to ensure relevance to a major aquaculture sector. The two strains tested were both originally isolated from diseased invertebrate larvae and reported as luminescent: *Vibrio sp.* ISO7 (personal communication) and *V. owensii* 47666-1 (Harris, 1993; Muir, 1991; Pizzutto and Hirst, 1995). Here, the strains were identified using molecular techniques,

luminescence was tested, and infection experiments were performed with prawns to confirm Koch's postulates.

2.2 Materials and Methods

2.2.1 Origin of *Penaeus monodon*

Penaeus monodon post-larvae (PL 55) were donated by Crystal Bay Prawn Farm in Cardwell, (North) Queensland, Australia. The experimental tanks (see 2.2.2) were 65 L glass aquaria filled with ~35 L of filtered and UV-sterilized seawater. Healthy-looking prawns of similar size (3 g) were randomly selected and divided into 3 groups of 4 animals and assigned to separate experimental tanks. Another 30 prawns were kept in captivity to be used for subsequent challenge experiments and transferred to a holding tank (65 L glass aquaria with lid, Blue Planet, Chicago, Illinois, USA) filled with ~50 L filtered and UV-sterilized seawater (MARFU, James Cook University, Townsville, Australia).

2.2.2 Aquaria Set up, Water Quality and Feeding

Aquaria were set up in a controlled temperature room (28 ± 1 °C) with a 12/12 h photoperiod in the North Queensland Algae Identification Facility (NQAIF, James Cook University, Townsville, Australia). Air pumps (4-outlets, Blue Planet) were used for aeration. To avoid cannibalism in the experimental tanks, tank dividers, made from plastic sliding bars and marine flyscreen, were used to separate the prawns. Prawns were fed twice daily with commercial feed pellets (Prawn MR Ridley diet, Ridley Aqua Feed, Melbourne, Victoria, Australia). To avoid nutrient build-up, aquaria were siphoned and water was batch exchanged (~75%) daily. Water quality (NH_4^+ , PO_4^{3-} , NO_3^{2-} and NO_2^{2-}) and pH was monitored daily using commercial aquaria kits (API, Aquarium Pharmaceuticals, Product #LR1800) and a pH meter (OAKTON, Eutech Instruments, Vernon Hills, Illinois, USA), respectively.

2.2.3 Bacterial Strain and Culture Media

The prawn challenge experiment was authorized under the Animal Ethics Approval Number A2062 by JCU's Animal Ethics Committee. The naturally bioluminescent *Vibrio* sp. strain ISO7 was originally isolated from diseased larvae of

the oyster *Saccostrea glomerata* in the Veterinary Science Laboratory at James Cook University in 2002. At the start of this study the strain had not been precisely identified using molecular methods and was referred to as *V. harveyi* ISO7 (personal communication). Its virulence had not been confirmed in infection studies.

Table 2.1: Media components.

Media	Components (g·L ⁻¹)
Artificial seawater (ASW) ¹	CaCl ₂ ·2H ₂ O (1.5), KCl (0.75), MgCl ₂ ·6H ₂ O (5.18), MgSO ₄ ·7H ₂ O (6.2) and NaCl (17.5)
Marine Basal Medium (MBM) ²	1M Tris buffer, pH 7.5 (20 mL), C ₆ H ₈ FeNO ₇ (0.025), K ₂ HPO ₄ (0.07) and NH ₄ Cl (1)
Luminescent broth Medium (LM) ³	Glycerol (3 mL), yeast extract (5) and trypton (5)
Luminescent Agar (LA) ⁴	Bacteriological agar (40)
Seawater Nutrient Medium (SWNM) ²	Yeast extract (5), trypton (1)
Seawater Nutrient Agar (SWNA) ⁵	Bacteriological agar (30)
Thiosulfate-Citrate-Bile-Sucrose (TCBS) agar	265020 - BD Difco™ TCBS Agar

¹ DI water (1L), ²ASW (1L), ³MBM (1L), ⁴LM (1L) or ⁵SWNM (1L)

The *V. owensii* 47666-1 isolate, was originally isolated from diseased prawn larvae (Harris, 1993), and demonstrated to be virulent to prawn larvae (Pizzutto and Hirst, 1995). This strain was described as a luminescent *V. harveyi* strain (Harris, 1993; Muir, 1991; Pizzutto and Hirst, 1995). More recently, it was identified as belonging to the new species *Vibrio owensii* using multi-locus sequencing (Cano-Gomez et al., 2010; Cano-Gomez et al., 2011) and whole genome sequencing (Espinoza-Valles et al., 2015).

Colony morphology and luminescence observations were performed for both strains using the following growth media: Luminous broth (LM), Luminous agar (LA), Seawater Nutrient broth Medium (SWNM), Seawater Nutrient agar (SWNA) and Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar (Table 2.1). Luminescence was recorded using a Vilber Lourmat Chemismart 3000 camera and Chemic Capt 3000 software (Eberhardzell, Germany).

2.2.4 Challenge Experiment 1 setup

Immediately before the experiment, both bacteria were streak-plated from cryo-preserved stocks onto TCBS agar, and incubated at 28 °C for 24 h. One colony of *Vibrio sp* ISO7 was resuspended in LM and the overnight culture (28 °C, 200 rpm) was diluted 100-fold in LM and incubated further until an OD₅₇₀ = 0.11 was reached

(Enspire 2300, Perkin Elmer, Waltham, Massachusetts, USA). Plating on LA showed that this OD corresponded to $\sim 1 \times 10^7$ CFU·mL⁻¹. Similarly, one colony of *V. owensii* 47666-1 was suspended in SWNM and incubated at 28 °C overnight (~ 18 h, 200 rpm). The culture was 100-fold diluted in SWNM and incubated (28 °C, 200 rpm) until an OD₅₇₀ = 0.22 was reached (Enspire 2300, Perkin Elmer, Waltham, Massachusetts, USA). This OD correspond to $\sim 1 \times 10^7$ CFU·mL⁻¹ when plated on SWNA.



Figure 2.1: Injection in the 3rd segment in *P. monodon*.

For each bacterium, 1 mL of the culture was centrifuged (Sigma 1-14, John Morris Scientific, Willoughby, NSW, Australia) at 10,000 g for 10 min and the pellet washed twice before resuspension in 1 mL of 2% NaCl Phosphate Buffer Saline (PBS) (Cano-Gomez, 2012). Sterile syringes with small needles (Terumo U-100 insulin, 1 mL 29 Gx1/2 inc, Tokyo, Japan) were used for intramuscular injections and to collect the haemolymph from dead prawns.



Figure 2.2: Example of haemolymph collection.

Prawns were acclimated for 48 h in the new environment before starting the experiment. One separate tank was used for each treatment (*Vibrio sp* ISO7, *V. owensii* 47666-1, 2% PBS control), with 4 prawns per tank. The freshly prepared bacterial

suspension (0.1 mL corresponding to $\sim 1 \times 10^6$ CFU·mL⁻¹) was intramuscularly injected into the third abdominal segment anterior to the telson (Figure 2.1) (Cano-Gomez, 2012; Harris and Owens, 1999). Control prawns were injected with 0.1 mL of sterile 2% NaCl PBS. The animals were monitored every hour for 6 h and then again after 12 h and every 12 hours afterwards. When death occurred, haemolymph was collected from the pleopod base of the first abdominal segment (Figure 2.2) (Vargasalbores et al., 1993). Control prawns were monitored for 7 days after injection and on Day 7 haemolymph of a control prawn was collected. Haemolymph from dead prawns and selected healthy control prawns were streaked on TCBS agar, incubated at 28 °C for 24 h and analysed for luminescent colonies as described above. One TCBS agar plates with haemolymph from a recently dead challenged prawn and a corresponding plate from a sacrificed control prawn were selected randomly, and colonies picked and purified.

2.2.5 Challenge Experiment 2 setup

The aim of the second experiment was to investigate if the pathogenicity of *V. owensii* 47666-1 could be stimulated after passage through prawns in the first experiment (Cano-Gomez, 2012; Egidius et al., 1986). The second challenge experiment was carried out immediately after the first experiment using prawns from the same batch as used in the first challenge experiment. Their total time in the holding tank before transfer to experimental tanks was 9 days followed by 48 h acclimations in experimental tanks as described above (2.2.4). A strain isolated from dead prawns in the first challenge experiment and identified as *V. owensii* using multiplex PCR (see 2.2.6), was cultured and injected into prawns as described for *V. owensii* 47666-1 in 2.2.4.

2.2.6 Multiplex PCR

A multiplex PCR protocol, that can identify and discriminate between the four closely related species *V. campbellii*, *Vibrio harveyi*, *V. owensii*, and *Vibrio rotiferianus* (Cano-Gomez et al., 2015), was used to screen bacteria isolated from both challenge experiments in order to select representative strains for sequencing. Cells picked from overnight colonies were suspended in 500 µL of TE (10 mL of 0.5 M Tris HCl + 1 mL of 0.5 M Na₂EDTA), and DNA was released by placing samples in boiling water for 10 min.

The PCR mastermix and cycling conditions were as described previously for DNA extracts (Cano-Gomez et al., 2015), except that the HotStar Taq DNA polymerase (Qiagen) concentration was adjusted to 0.035 U μL^{-1} and the final reaction volume was 20 μL . Controls consisted of purified DNA of *Vibrio fortis* (negative control), PCR water (blank), as well as a mixture of purified DNA from *V. campbellii*, *V. harveyi*, *V. owensii* and *V. rotiferianus* (positive controls). PCR products were separated on a 2.5% agarose gel with ethidium bromide (0.5 $\mu\text{g mL}^{-1}$, MO BIO Laboratories, Carlsbad, CA, USA) at 80 V for 80 min (Figure 2.4, Figure 2.6 and Figure 2.7).

Table 2.2: Sequence accession numbers of target genes from selected type-strains (T) and strains previously identified by multilocus sequence analysis (MLSA).

Type-strain	topA	mreB
<i>Photobacterium. damsela</i> subsp. <i>damsela</i> ATCC33539 ^T	DQ907458	DQ907386
<i>Photobacterium. damsela</i> subsp. <i>damsela</i> JCM8968 ^T	DQ907460	DQ907388
<i>Photobacterium phosphoreum</i> IAM14401 ^T	DQ907465	DQ907393
<i>Vibrio alginolyticus</i> LMG4409 ^T	DQ907472	DQ907405
<i>Vibrio campbellii</i> LMG11216 ^T	DQ907475	DQ907408
<i>Vibrio campbellii</i> LMG11257 ¹	JF930502	JF930413
<i>Vibrio campbellii</i> LMG21362 ¹	JF930493	JF930418
<i>Vibrio campbellii</i> Oz07 ²	HQ449879	HQ449951
<i>Vibrio harveyi</i> LMG4044 ^T	DQ907488	DQ907422
<i>Vibrio owensii</i> DY05 ^T	GU111255	GU111259
<i>Vibrio owensii</i> 47666-1 ³	GU111254	GU111258
<i>Vibrio parahaemolyticus</i> LMG2850 ^T	DQ907509	DQ907440
<i>Vibrio rotiferianus</i> LMG21460 ^T	DQ907515	DQ907445

¹ Hoffmann et al. (2012); ² Cano-Gomez et al. (2011); ³ (Cano-Gomez et al., 2010)

2.2.7 Gene Sequencing

For selected strains, one colony was used to inoculate a 5 mL culture in alkaline peptone water (APW; Peptone 1%, NaCl 3%, MQ water, pH 8.5) and incubated overnight (28°C, 200 rpm). Extraction of DNA from overnight cultures was performed using the Wizard® Genomic DNA Purification Kit (Promega) following the manufacturer's guidelines for Gram-negative bacteria. For each strain, two different PCRs were performed; one to amplify the topA gene and one to amplify the mreB gene. The PCR mastermix and cycling conditions were as described previously (Cano-Gomez et al., 2011), except that the primer concentrations were adjusted to 0.4 μM , the HotStar Taq DNA polymerase (Qiagen) concentration was adjusted to 0.05 U μL^{-1}

and the final reaction volume was 50 μL . The PCR products were electrophoresed on a 1% agarose gel with ethidium bromide ($0.5 \mu\text{g mL}^{-1}$, MO BIO Laboratories, Carlsbad, CA, USA) for 80 min at 80 V to confirm that fragments of the expected size were amplified for samples and positive controls only; milli-Q water was used as a negative control (Figure A2.1, Figure A2.2 and Figure A2.3). Samples were sent to Macrogen Inc. (Seoul, Republic of Korea) for sequencing in both directions using the same primers as for the original amplification reactions.

2.2.8 Sequence Analysis and Phylogenetic Tree

Gene sequences were analysed, aligned and phylogenetic trees constructed using CLC Main Work bench 7.6. For each sample, contigs of the *mreB* and *topA* sequences were created. The corresponding sequences of selected type strains (Table 2.2) were obtained from the NCBI website. Sequences were aligned using CLUSTAL v2.1 Multiple Sequence Alignments and a phylogenetic tree constructed using the Maximum Likelihood function (Figure 2.5 and Figure 2.8).

2.3 Results and Discussion

Both *Vibrio sp.* ISO7 and *Vibrio owensii* 47666-1 grew in all the tested media (Figure 2.3), but bioluminescence was only observed for *Vibrio sp.* ISO7 (data not shown). While *V. owensii* 47666-1 was originally described as luminescent (Harris, 1993; Muir, 1991; Pizzutto and Hirst, 1995), this was not confirmed in more recent studies by (Cano-Gomez et al., 2010) and in the current study.

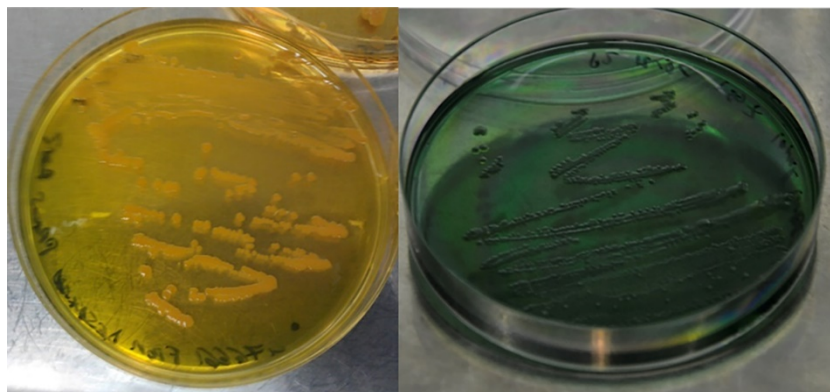


Figure 2.3: *V. owensii* 476661 (left) and *V. harveyi* ISO7 (right) streak-plated in TCBS agar. *V. owensii* 47666-1, a sucrose-fermenter, produced yellow colonies. *Vibrio sp.* ISO7, on the other hand, is a non-sucrose-fermenting strain that produces green colonies.

2.3.1 Challenge Experiment 1

Vibrio sp ISO7 induced 100% mortality between 6 and 16 h after injection, while control prawns injected with sterile 2% NaCl PBS remained healthy. This demonstrates that the buffer itself was non-toxic and animals were not affected by handling procedures. In contrast, in challenge experiments with *V. owensii* 47666-1, one prawn died within 1 h after injection, most likely due to stress and not bacterial pathogenicity, one prawn died between 6 and 16 h after injection, and 2 prawns remained healthy for the entire experiment (7 days). These results are comparable to those from Cano-Gomez (2012), where *V. owensii* 47666-1 was shown to have limited virulence in *P. monodon* juveniles, but in contrast to Harris (1993) and Pizzutto and Hirst (1995), who described this specie as highly virulent towards prawn larvae. It is also possible that the life stage of the animals influenced disease susceptibility since juveniles do not possess an adaptive immune system (Hauton et al., 2015; Kumaresan et al., 2016; Rowley and Pope, 2012). Alternatively, the pathogenicity of the bacterium might have been lost or reduced after multiple passages in artificial culture media and a long cryo-preservation time. In the latter case, virulence may be restored through multiple passages of the bacterium in the original host (Egidius et al., 1986).

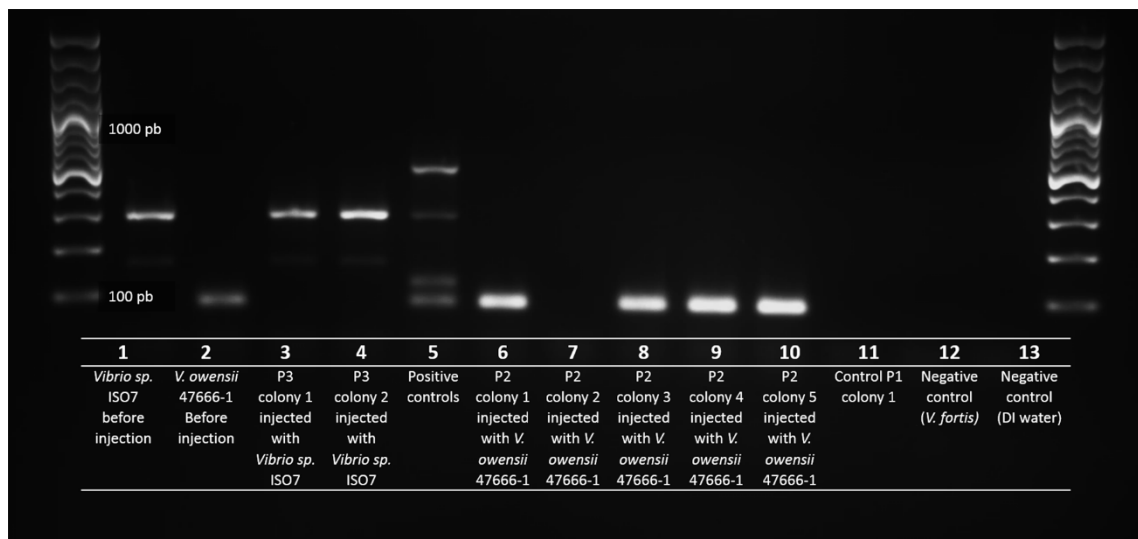


Figure 2.4: Multiplex PCR challenge experiment 1. Colonies picked from plated haemolymph of dead (P1 to P4) or sacrificed (control) prawns. Positive control consists of (from the bottom to top) *V. owensii* (85 bp), *V. harveyi* (121 bp), *V. campbellii* (294 bp), and *V. rotiferianus* (489 bp); DNA ladder (100 bp) on the edge of the gel.

Multiplex PCR from the first experiment suggested that *Vibrio sp* ISO7 belongs to the *V. campbellii* species (Figure 2.4: *Vibrio sp* ISO7 lanes 1, 3 and 4) and that *V.*

owensii 47666-1 belongs to the *V. owensii* species (Figure 2.4: *V. owensii* 47666-1 lanes 2, 6, 8, 9 and 10). Indeed, the original strain and all the isolates from injected prawns with *Vibrio* sp ISO7 or *V. owensii* 47666-1 produced an amplification product at a site expected for *V. campbellii* (Figure 2.4: *Vibrio* sp ISO7 lanes 1, 3 and 4) or *V. owensii* (Figure 2.4: *V. owensii* 47666-1 lanes 2, 6, 8, 9 and 10), respectively. The identity was confirmed by using phylogenetic tree analysis (Figure 2.5) since the strains clustered with the *V. campbellii* or *V. owensii* type strains. These results in combination with the phylogenetic tree analysis fulfil Koch's postulate (Fredricks and Relman, 1996; Rivers, 1937).

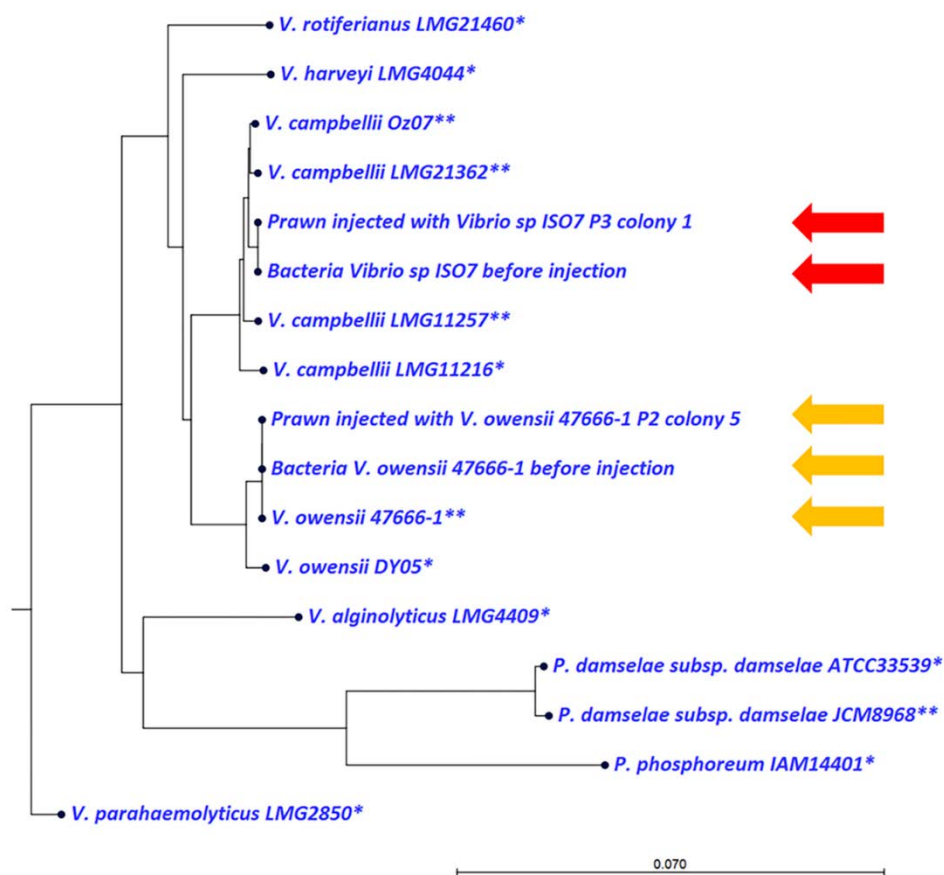


Figure 2.5: Phylogenetic tree using concatenated *topA* and *mreB* gene sequences from selected type strains (*; Table 2.2), reliable strains (**; Table 2.2) and colonies collected from challenge experiment 1. Red arrows show the position of *V. campbellii* ISO7 before the injection and after successful recollection from dead prawns. Yellow arrows point to *V. owensii* before injection, and after successful recollection from dead prawns and the reference sequence of *V. owensii* 47666-1 (Table 2.2), to which both were identical.

Strains isolated from a sacrificed control prawn (Figure 2.4: lane 11) did not produce an amplification product in the Multiplex PCR, confirming bacteria present were not identical to any of the four-target species (*V. owensii*, *V. harveyi*, *V.*

campbellii, and *V. rotiferianus*; Figure 2.4: lane 5) and demonstrating that the injected strains were not present in the control prawns. In addition, one colony from a prawn injected with *V. owensii* 47666-1 (Figure 2.4: lane 7) did not amplify suggesting that the colony selected (randomly) belonged to a bacterial species different to the four-target species investigated (*V. owensii*, *V. harveyi*, *V. campbellii*, and *V. rotiferianus*; Figure 2.4: lane 5).

In conclusion, these results showed that the same strain could be reisolated from dead individuals after challenge experiments confirming Koch's postulates. Gene sequences of the topA and mreB genes from *Vibrio campbellii* ISO7 have been submitted to GenBank, with accession numbers KY11.

2.3.2 Challenge Experiment 2

A colony isolated from a prawn that died 6 h after injection in the first challenge experiment and identified as *V. owensii* by Multiplex PCR (Figure 2.4: lane 10) was purified and re-injected in *P. monodon*. Subsequently, 2-loci MLSA confirmed that the purified strain was *V. owensii* 47666-1 (Figure 2.5).

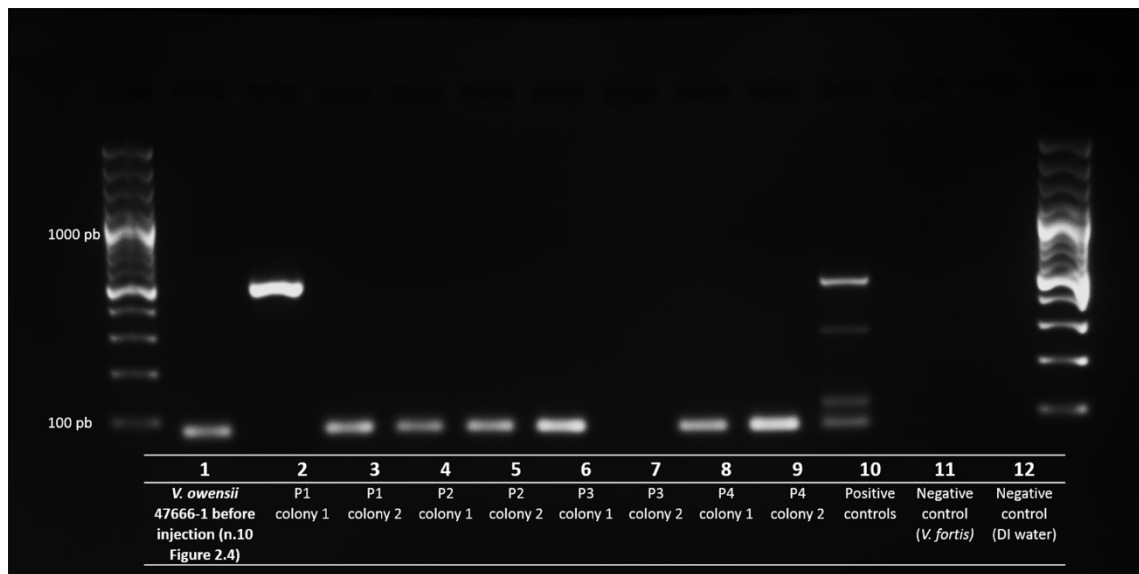


Figure 2.6: Multiplex PCR challenge experiment 2 (injected prawns). Alive prawns (P1 to P4) were intramuscularly injected with *V. owensii* 47666-1 and colonies were picked from plated haemolymph of dead prawns. Positive control consists of (from the bottom to top) *V. owensii* (85 bp), *V. harveyi* (121 bp), *V. campbellii* (294 bp), and *V. rotiferianus* (489 bp); DNA ladder (100 bp) on the edge of the gel.

While all injected prawns died during the experimental period, there was a large variability in time to mortality (1 h, 6-16 h, 6-16 h and 6 days). Colonies isolated

from prawns injected with *V. owensii* 47666-1 that died 6-16 h after injection confirmed the amplification product to be identical to *V. owensii* by multiplex PCR (Figure 2.6) and clustering with the reference *V. owensii* type strain in the phylogenetic tree (Figure 2.8). Therefore, Koch's postulate was successfully fulfilled and new stock of *V. owensii* 47666-1 was cryopreserved in 30% glycerol.

In addition, in this experiment one of the control prawns died after injection. Multiplex PCR, revealed that colonies isolated from hemolymph of control prawns belonged to *V. harveyi* (Figure 2.7: lanes 1, 2, 3, 4 and 5) showing that likely, stress condition due to long captivity (> 9 days) created favourable conditions for opportunistic bacterial to grow and causing death.

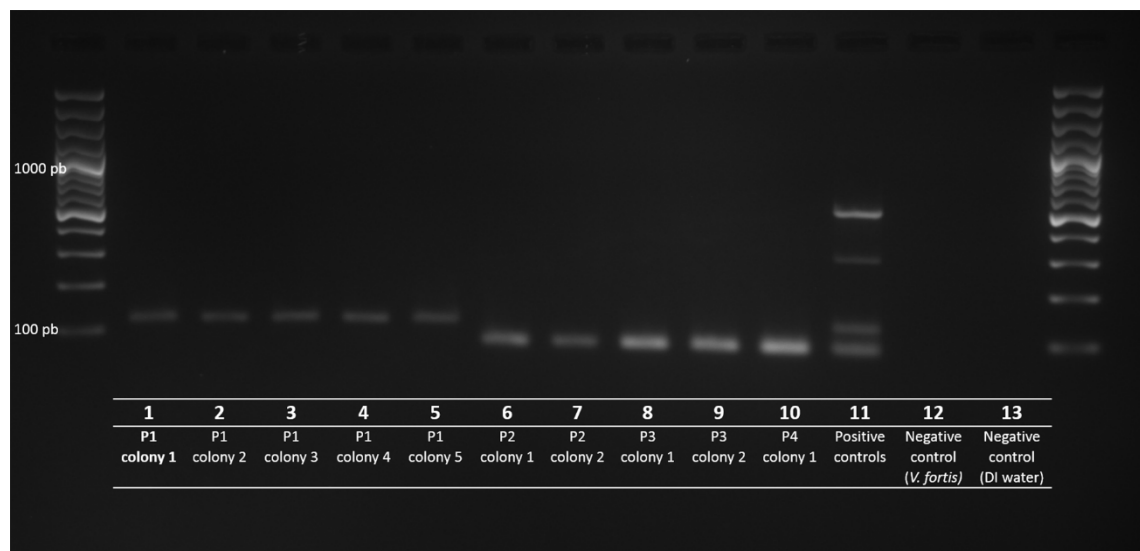


Figure 2.7: Multiplex PCR challenge experiment 2 (control prawns). Colony picked from plated haemolymph of control prawns (P1 to P4). Positive control consists of (from the bottom to top) *V. owensii* (85 bp), *V. harveyi* (121 bp), *V. campbellii* (294 bp), and *V. rotiferianus* (489 bp); DNA ladder (100 bp) on the edge of the gel.

We also noticed that *V. owensii* (Figure 2.7: lanes 6, 7, 8, 9 and 10) was present in the healthy control prawns that were sacrificed. However, the phylogenetic tree (Figure 2.8) based on 2-loci MLSA showed that those colonies although belonging to *V. owensii* group but were not identical to the injected *V. owensii* 47666-1. The hypothesis is that prawns already had this bacterium in their tissues/hemolymph and it proliferated while prawns were kept in holding tanks or that it was introduced into the tank during water exchanges.

In conclusion, these results suggest that even in the second experiment the injected bacterium was successfully re-isolated from the diseased prawns, fulfilling

Koch's postulates. Occurrence of bacteria belonging to the *V. owensii* group in the second challenge experiment suggests that these bacteria can proliferate if animals are kept in holding tanks for prolonged times, which may affect survival of control animals. It was also evident that the second challenge experiment did not increase the boost effect leading to the conclusion that *V. campbellii* ISO7 was the best candidate for a model bacterium due to natural luminescence and potential pathogenicity towards *P. monodon*.

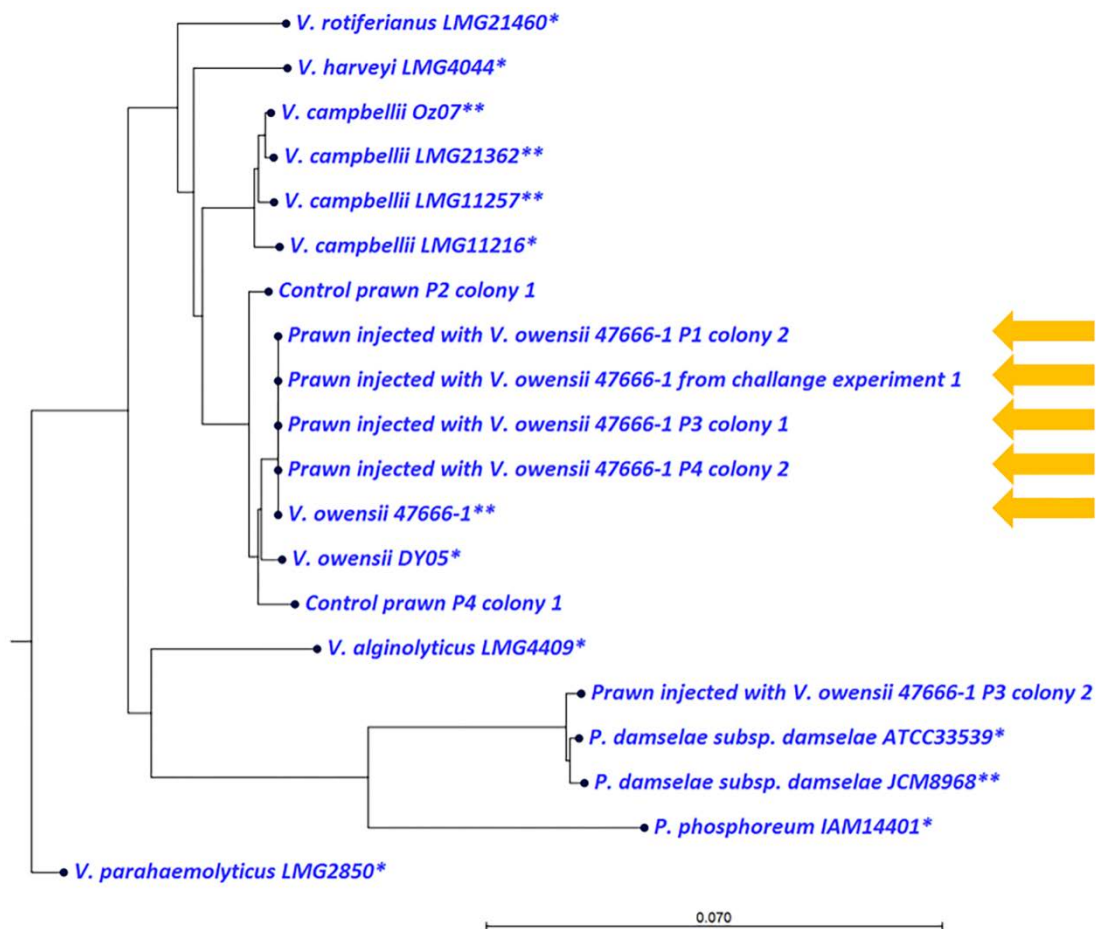


Figure 2.8: Phylogenetic tree using concatenated topA and mreB gene sequences from selected type strains (*; Table 2.2), reliable strains (**; Table 2.2) and colonies collected from challenge experiment 2. Yellow arrows indicated the *V. owensii* before the injection was successfully recollected from dead prawns and the sequence was identical to the reference sequence of *V. owensii* 47666-1 (Table 2.2).

2.4 Summary

The aim of this study was to identify whether *Vibrio* sp ISO7 or *V. owensii* 47666-1 were suitable model prawn pathogens, in order to use a reliable and well characterised *Vibrio* prawn pathogen in the subsequent chapters of this thesis. Both

strains were originally reported as luminescent, *Vibrio sp.* ISO7 was isolated from diseased oyster larvae while *V. owensii* 47666-1 strain was isolated from diseased prawn larvae. The infection experiment successfully caused disease in injected animals. The results from multiplex PCR and gene sequencing confirmed that Koch's postulates were fulfilled for *Vibrio sp.* ISO7 (previously *V. harveyi* ISO7) and *V. owensii* 47666-1, with bacteria injected and those collected from dead prawns being identical. In addition, *Vibrio sp.* ISO7 was identified as belonging to *V. campbellii* species and showed a natural bioluminescence while *V. owensii* 47666-1 was not luminescent. The *V. campbellii* ISO7 strain was more highly virulent in the first experiment when compared to *V. owensii* 47666-1. Indeed, *V. owensii* 47666-1 required a second infection experiment. The boosting step successfully produced a sub-culture with enhanced virulence relative to the original cryo-preserved stock culture but *V. owensii* 47666-1 continued to be not as virulent as *V. campbellii* ISO7. In the light of these results and the aim of this experiment, *V. campbellii* ISO7 was chosen as model bacterium for the following chapters.

3 Chapter 3: ³Capacity of Cationic and Anionic Porphyrins to Inactivate the Prawn Pathogen *Vibrio campbellii*

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3.1 Introduction

Aquaculture water is considered one of the most important carriers of microbial pathogens (Meyer, 1991). Currently, direct photolysis (UV sterilization), ozonation (O₃) and chlorination (Cl₂) are the most widely used water sterilization treatments (Acher et al., 1997; Jensen et al., 2011; Jorquera et al., 2002). Despite their efficiencies, these techniques all have a variety of drawbacks, including the handling of large volumes of health- and environmentally hazardous solutions (Cl₂), production of toxic by-products (O₃) (Hall et al., 2013; Jensen et al., 2011), and high energy requirements (UV and O₃). To make aquaculture more environmentally and economically sustainable, novel water treatment technologies are therefore urgently required. An alternative to traditional sterilization methods is photodynamic antimicrobial chemotherapy (PACT), which relies on the use of catalytic amounts of photosensitisers (PS), light and dissolved oxygen. Photosensitisers can be synthetic or natural compounds (Alves et al., 2015b), typically dyes, that when exposed to light and oxygen create ROS (Alves et al., 2015b; Arrojado et al., 2011; Carvalho et al., 2009; Jori

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and Coppellotti, 2007; Kanyal et al., 2016; Maisch, 2015a; Sabbahi et al., 2013; Sperandio et al., 2013; Vatansever et al., 2013; Yoon et al., 2013), which through multi-target action on cellular macromolecules (i.e. lipopolysaccharides, proteins and fatty acids), damage organisms leading to death (Alves et al., 2015a; Alves et al., 2015b; Bacellar et al., 2015; Benov, 2015; Stojiljkovic et al., 2001). Porphyrins produce mainly $^1\text{O}_2$ as ROS but it depends on the experimental condition (Alves et al., 2015b; Arrojado et al., 2011; Carvalho et al., 2009; Jori and Coppellotti, 2007; Kanyal et al., 2016; Maisch, 2015a; Sabbahi et al., 2013; Sperandio et al., 2013; Vatansever et al., 2013; Yoon et al., 2013). Due to the indiscriminate action of $^1\text{O}_2$, acquisition of resistance *per se* is not possible for PACT (Tavares et al., 2010). However, sub-lethal $^1\text{O}_2$ treatments activate reactive oxygen defence mechanisms in bacteria (Maisch, 2015b), making them more tolerant to $^1\text{O}_2$ levels. Bacteria are generally more vulnerable to $^1\text{O}_2$ compared to mammalian cells (Demidova and Hamblin, 2004; Hamblin and Hasan, 2004; Jori and Coppellotti, 2007), enabling the use $^1\text{O}_2$ as antimicrobial tools. PACT-generated $^1\text{O}_2$ has been shown to destroy virulence factors and can be classified as an antimicrobial and anti-virulence factor therapy (Hamblin and Hasan, 2004; Komerik et al., 2000).

Photosensitizers are divided into cationic, anionic or neutral, based on the presence of positive, negative or neutral charges, respectively (Stojiljkovic et al., 2001). Porphyrins are a sub-group of PS that often photobleach rapidly, thereby limiting the duration of their activity ($^1\text{O}_2$ generation) and making them environmentally-friendly and ideal in applications for environmental studies and aquaculture (Alves et al., 2011b). Many types of porphyrins have been synthesized and tested (Castano et al., 2004; Nyman and Hynninen, 2004; Oliveira et al., 2009; Philippova et al., 2003). With regards to effectiveness and compound charge, cationic porphyrins appear advantageous for eradicating Gram-negative bacteria (Alves et al., 2015b; Ragas et al., 2013; Stojiljkovic et al., 2001) compared to anionic and neutral porphyrins, however, some exceptions have been demonstrated previously (Malara et al., 2017b). Antimicrobial efficiency of PACT using porphyrins (i.e. cationic) seems to depend on number of charges (Lazzeri et al., 2004) and the proximity to the target organism, with best results obtained if the dye electrostatically interacts with the outer membrane of

Gram-negative bacteria and when taken up by the cells (Alves et al., 2015b; Benov, 2015; Gsponer et al., 2015; Wikene et al., 2015).

While the bulk of studies on the use of PS have targeted their potential use in cancer treatment, there have been some encouraging results for the eradication of water-borne pathogens in wastewater and drinking water (Bonnett et al., 2006; Carvalho et al., 2007; Carvalho et al., 2009; Jemli et al., 2002). Only a few studies to date have explored the potential use of PACT in an aquaculture context (Alves et al., 2011b; Arrojado et al., 2011; Magaraggia et al., 2006; Schrader et al., 2010). In 2006, Magaraggia et al. (2006) explored the use of PACT for preventative and curative treatment of the freshwater mould *Saprolegnia* spp on rainbow trout. Later, in 2010, Schrader et al. (2010) tested the antibacterial activity of a patented, commercially available porphyrin product *in vitro* against bacteria that are pathogenic to channel catfish, *Ictalurus punctatus*. Two studies have explored the use of porphyrins in PACT in anti-microbial treatments in seawater (Alves et al., 2011b; Arrojado et al., 2011). Alves et al. (2011b) used the naturally luminescent marine bacterium *Vibrio fischeri* to demonstrate that a tri-cationic porphyrin could photoinactivate *V. fischeri* under a range of different physico-chemical conditions. In addition, Arrojado et al. (2011) showed the effect of a tri-cationic porphyrin on reducing cell numbers for a range of marine bacteria including both Gram-negative and Gram-positive species in phosphate buffered saline (PBS). Recently, our group tested the efficacy of photolytic and photodynamic disinfection protocols using one tetra-cationic and one neutral porphyrin and two pathogenic *Vibrio* species, *Vibrio parahaemolyticus* and *Vibrio owensii* in marine broth used for their cultivation (Malara et al., 2017b).

The main aims of this study were to effectively sterilize prawn hatchery water using porphyrin-enabled PACT. To achieve the main aim, the objectives were to test 1) suitability of the tetra-cationic porphyrin 5, 10, 15, 20-Tetrakis (1-methyl-4-pyridinio) porphyrin tetra (p-toluenesulfonate) (TMPyP) and the tetra-anionic porphyrin 4, 4', 4'', 4'''-(Porphine-5, 10, 15, 20-tetrayl) tetrakis (benzenesulfonic acid) tetrasodium salt hydrate (TPPS₄) in direct aquaculture applications via photobleaching experiments and 2) efficacy of both porphyrins to photoinactivate and kill the naturally luminescent

bacterium *Vibrio campbellii* ISO7 when suspended in seawater collected from an aquaculture farm.

Vibrio campbellii ISO7 belongs to the Harveyi-clade (Urbanczyk et al., 2013), which include many strains that cause luminescent vibriosis (Busico-Salcedo and Owens, 2013; Wang et al., 2015) and heavy financial losses in the aquaculture industry worldwide (Cano-Gomez et al., 2015; Defoirdt et al., 2007b; Defoirdt and Sorgeloos, 2012; Karunasagar et al., 1994). The bacterium was chosen as model for this experiment based on results presented in chapter 2 (section 2.3 for details). The photobleaching experiment used absorbance as a measure to detect the degradation of the porphyrins over 24 h. Detailed photoinactivation experiments were also carried out, using four different methods to evaluate the treatment effect on the bacterium in order to discriminate between inactivation and eradication.

3.2 Materials and Methods

3.2.1 Porphyrins

Two water soluble porphyrins were chosen for the PACT experiments: the tetra-cationic 5,10,15,20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (p-toluenesulfonate) [TMPyP] (Product # 323497, Sigma-Aldrich, Castle Hill NSW, AUSTRALIA) (Figure 3.1a) and the tetra-anionic 4,4',4'',4'''-(porphine-5,10,15,20-tetrayl) tetrakis (benzenesulfonic acid) tetrasodium salt hydrate [TPPS4] (Product # 88074, Sigma-Aldrich) (Figure 3.1b). For each porphyrin, a stock solution of 500 μM was prepared in 100% dimethyl sulfoxide (DMSO).

3.2.2 Bacterial strain and growth conditions

The naturally luminescent bacterium *V. campbellii* ISO7 was streak-plated on LA from cryo-preserved stock and incubated at 28°C as described below (Section 3.2.3). After 24 h, one colony was resuspended in 30 mL of LM and grown for 12 h (28°C, 200 rpm). Afterwards, the culture was diluted 1:1 x 10⁶ in LM to a final volume of 10 mL and grown under the same conditions for up to 11 h. Optical density was measured at 570 nm (OD₅₇₀) in a 96-well plate (Isoplate^{TM-96} TC, Perkin Elmer, Product # 6005070) and the OD₅₇₀ was adjusted to ~0.3 using sterile LM (Multimode plate reader, Enspire 2300, Perkin Elmer). The OD-adjusted culture was diluted 10-fold in aquaculture water

containing porphyrins to a final concentration of bacteria of $\sim 1 \times 10^7$ CFU·mL⁻¹. The cell concentration at the start of the experiment (T0) was verified by streak-planting (n=3) each sample on LA plates.

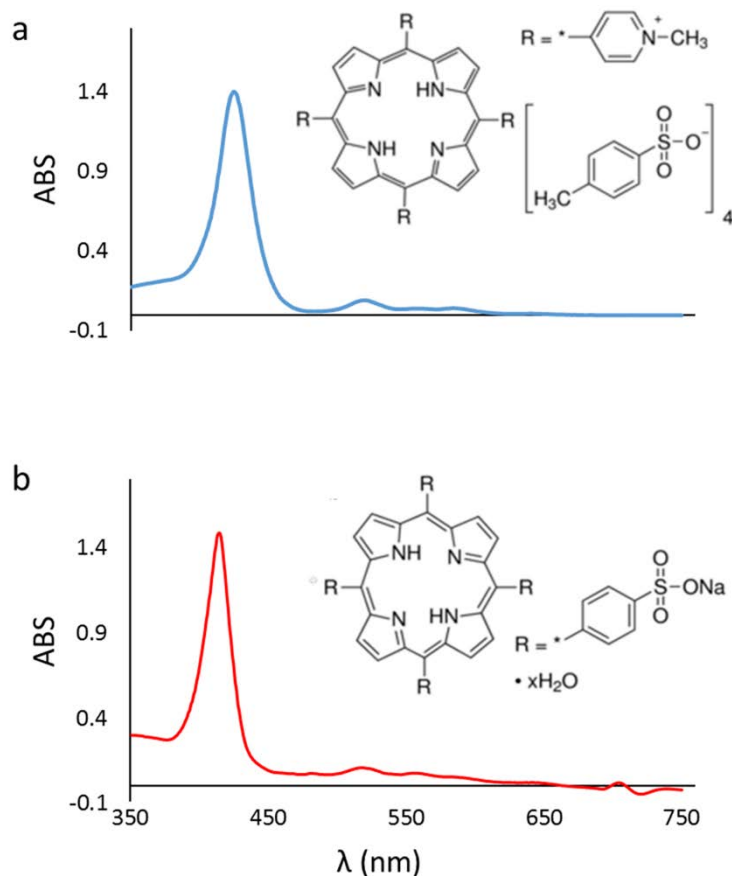


Figure 3.1: Molecular structures and ABS scans (350-750 nm) of 20 μ M TMPyP (a) and TPPS₄ (b) porphyrins in aquaculture water.

3.2.3 Experimental setup

Identical experimental setups were used to evaluate both photobleaching properties and phototreatment. Seawater was collected from the prawn hatchery at Crystal Bay Prawn Farm (18°16'04.9"S 146°01'48.8"E). The seawater was sand-filtered (1 μ m), ozone-sterilized (ORP reading > 700 mV), passed through a carbon filter followed by a 1 μ m polishing filter before UV sterilization. The water was autoclaved immediately prior to the start of experiments.

Porphyrins were diluted in autoclaved seawater to obtain 0, 1, 5, 10 and 20 μ M final concentrations at a bacterial concentration of $\sim 1 \times 10^7$ CFU·mL⁻¹. Each sample (4 mL) was placed in a 12 well tissue culture plate (Beckton Dickinson, Franklin Lakes, New Jersey, USA, product # 353043) (n=3 independent samples per porphyrin

concentration). To account for possible effects of light only or porphyrin only, light controls (0 μM porphyrin) and dark controls (20 μM porphyrin, plates wrapped in aluminium foil) were irradiated at the same time. Blank samples (including light and dark controls) were created using the same concentrations of porphyrins but sterile LM was added instead of the bacterial inoculum.

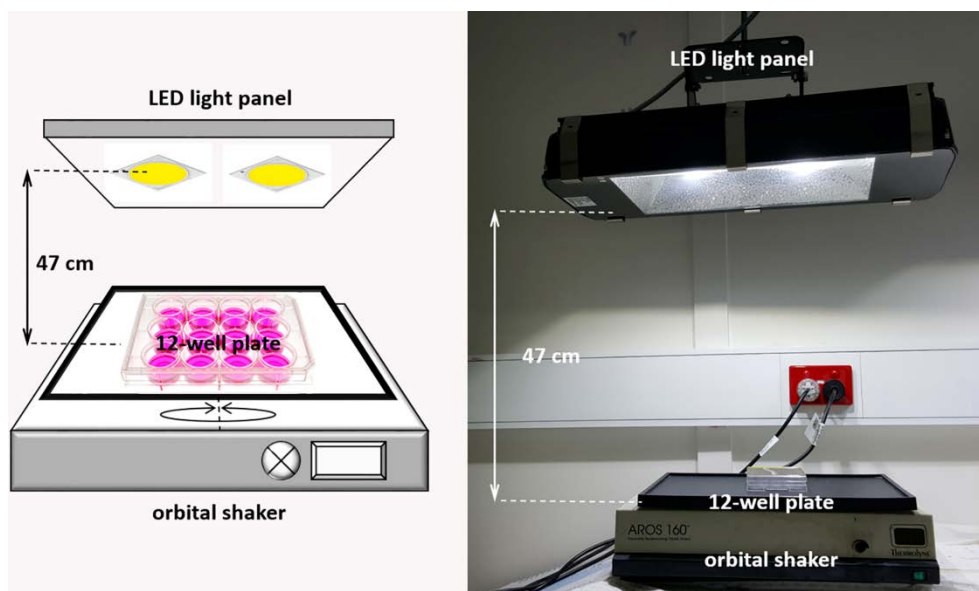


Figure 3.2: Experimental set-up: schematic (a) and example of well-plate under continuous illumination on an orbital shaker (b).

Samples, controls and blanks were incubated in the dark for 20 min under continuous agitation (150 rpm; AROS 160, Thermolyne, Thermo Fisher Scientific, Waltham, USA) to allow porphyrins to bind bacterial cells. Successively, they were irradiated for 24 h with high power 150 W cool white LED floodlights (with an emission spectrum between 400 and 700 nm and characterised by a large narrow emission peak at 465 nm and a broad emission peak from 500-700 nm) positioned 47 cm above the samples (Figure 3.2). Samples were exposed to a photon flux density of $223.8 \mu\text{mol m}^{-2}\text{s}^{-1}$ as measured using a light meter (LI-250, Li-Core, Lincoln, Nebraska, USA). This corresponded to 1.179 mW cm^{-2} based on the conversion reported by Alves et al. (2015b): $9.5 \times 10^{-3} \text{ mW cm}^{-2} \approx 1.8 \mu\text{mol m}^{-2}\text{s}^{-1}$. The well plates were placed on an orbital shaker (AROS 160, Thermolyne, Thermo Fisher Scientific) at 150 rpm to keep the samples homogeneously suspended.

3.2.4 Photobleaching evaluation

For both porphyrins, samples without bacteria (sterile) were scanned over the full light spectrum (350-750 nm) after 0, 6, 12 and 24 h to evaluate possible photobleaching of the porphyrin and possible water evaporation. Subtraction of the seawater absorbance was necessary to investigate porphyrin degradation.

3.2.5 Bacterial viability evaluation

Four different methods were used to evaluate the activity and viability of *V. campbellii* ISO7: luminescence signal, absorption at 570 nm (ABS_{570}), CFU counts and a regrowth assay. Samples (150 μ L) were collected and transferred to white framed, clear bottom 96 well plates (Isoplate^{TM-96} TC, Perkin Elmer, Product # 6005070). The ABS_{570} and luminescence (Relative Light Units (RLU)) were determined every 30 min up to 6 h and then at time 12 h and 24 h using a plate reader (Multimode plate reader, Enspire 2300, Perkin Elmer). Before the luminescence reading, a white back seal (BackSeal-96/384, PerkinElmer Pty Ltd, cat. # 6005199) was added to the plate. CFU counts and regrowth assays were carried out to discriminate between photoinactivation and eradication of *V. campbellii* ISO7. CFU counts were obtained by streak-plating 100 μ L of sample in triplicate on LA and incubating the plates at 28°C for 24 h. Regrowth assays were performed in triplicate by inoculating 100 μ L of sample into 1 mL LM and incubating the tubes at 28°C, 200 rpm for up to 7 days as described previously (Malara et al., 2017b). For both porphyrin experiments, CFU counts and regrowth assays were carried out at the start of the experiment (time 0) and as soon as the luminescence detection limit was reached for a sample and at every time point thereafter.

3.2.6 Statistical analysis

Statistical analyses were performed using R-Studio (RStudio Team, 2015) setting the significance level $\alpha=0.05$.

For the photobleaching (Figure A3.1 : Figure A3.4; Table A3.1 :Table A3.4), photoinactivation (Figure A3.5 : Figure A3.8; Table A3.5 : Table A3.8) and absorbance experiments (Figure A3.9 and Figure A3.10; Table A3.9 and Table A3.10), normality and equal variance of data were assessed using the Shapiro-Wilk test and Levene's test

(*Rcdm* package, v.2.3) (Fox, 2005, 2007), respectively. The analysis of variance (ANOVA) or Kruskal-Wallis' test (*stats* package, v.3.2.5) (Hollander and Wolfe, 1973; R Core Team, 2015) was used to detect significant differences between treatments and/or time.

In the photoinactivation experiment, Dunnett T3 test, which is a modified Tukey-Kramer pairwise multiple comparison post-hoc test adjusted for unequal variances and unequal sample sizes, was used to identify which treatments and/or time points were significantly different (*DTK* package, v.3.5) (Dunnett, 1980; Lau, 2013).

For the luminescence data, a time-course model was created using treatments (porphyrin concentration) as a grouping factor (*drc* package, v.3.0.1) (Ritz et al., 2015; Ritz and Streibig, 2016; Ritz and Streibig, 2005). We used the "mselect" function to select the best fitting model based on the Akaike Information Criterion (AIC) (Ritz et al., 2015). Time effects were determined by comparing the time-course model with a simple linear regression model with slope 0 (no time effect) using a Chi-square (X^2) test (Ritz et al., 2015). Lastly, the created model, was used to determine the time necessary to obtain 50% of luminescent inhibition (hereafter T-IC₅₀) and relative potencies (Ritz et al., 2015; Ritz et al., 2006). The relative potencies of different treatments (ratio between pairwise T-IC₅₀ values) were used to compare the effectiveness of treatments.

3.3 Results and Discussion

3.3.1 Photobleaching and photoinactivation experiments

Photobleaching of porphyrins can be beneficial in aquaculture water treatments containing farmed animals, as the build-up of porphyrins in animal tissues would be avoided. The photostability of TMPyP and TPPS₄ was affected by irradiation showing a time-dependent photobleaching effect over 24 h light exposition (TMPyP: Figure 3.3 and TPPS₄: Figure 3.4, respectively). As expected, due to the absence of light, the dark control did not photobleach showing continued high absorption at the respective absorption maximum (TMPyP: 425 nm in Figure 3.3; TPPS₄: 414 nm in Figure 3.4). A slight increase in absorption with time was apparent in the dark control, which could be due to evaporation caused by continuous irradiation (temperature under

LEDs $\sim 25.5^{\circ}\text{C}$) and relatively small volumes of sample (4 mL). It was less evident in TMPyP, where time points were found not significant different (Kruskal-Wallis $\chi^2 = 2.28$, $\text{df} = 3$, $p = 0.52$), than in TPPS₄ (Kruskal-Wallis $\chi^2 = 9.58$, $\text{df} = 3$, $p = 0.02$) where, at the end of the experiment, a slightly higher increase in absorbance in the dark control was observed. The light control did not absorb at the used wavelength (data not shown), as it contained no porphyrin confirming that the wavelengths used for detection of TMPyP or TPPS₄ were specific. The photobleaching properties supported the potential use of both porphyrins in aquaculture, as they remained non-degraded in solution for a limited time avoiding any accumulation in the environment or possibly in animals.

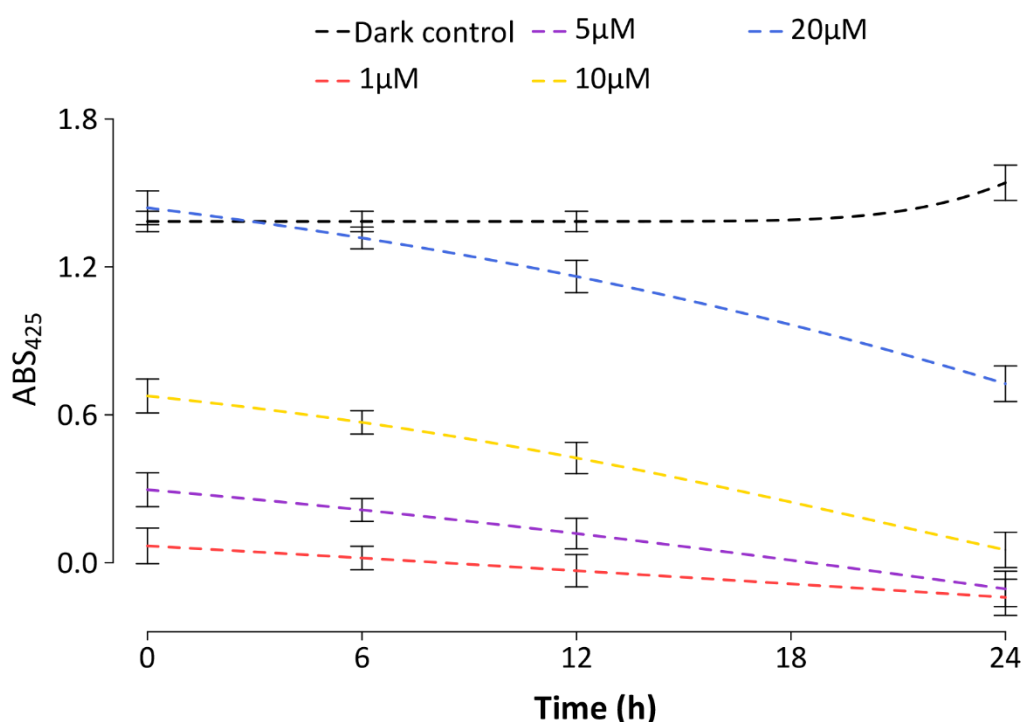


Figure 3.3: 24 h-time-course of photobleaching of the TMPyP porphyrin determined at the absorption maximum of 425 nm. Error bars show model-based standard errors ($n=3$) (Ritz and Streb, 2016).

Bacterial luminescence has been successfully used in ecotoxicological assays (Alves et al., 2011a; De Zwart and Slooff, 1983) and has been described as a rapid tool to assess bacterial metabolic activity (Alves et al., 2011a; Demidova et al., 2005). However, bacterial luminescence depends on a range of environmental variables including nutrient and oxygen availability, which limits the incubation period as the luminescence signal is also ultimately reduced in untreated controls. Moreover, although reduction of bioluminescence below the detection limit is correlated with a

loss of bacterial activity, it does not provide unambiguous evidence regarding the lethality of the treatment. In the current study, we therefore included additional assays to also investigate the effect of porphyrin treatment on bacterial growth activity in seawater (ABS_{570}) and on agar plates (CFU counts), as well as an assay to investigate if growth in liquid culture could recover within 7 days (regrowth assay).

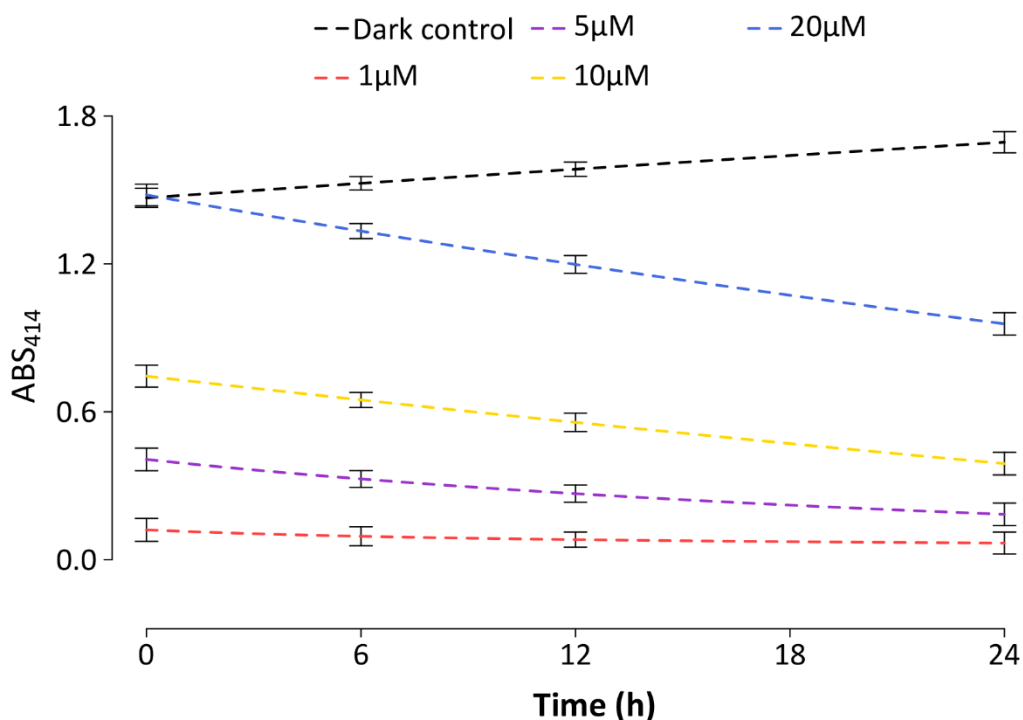


Figure 3.4: 24 h-time-course of photobleaching of the TPPS₄ porphyrin determined at the absorption maximum of 414 nm. Error bars show model-based standard errors (n=3) (Ritz and Strebig, 2016).

Table 3.1: Time at which the luminescent signal decrease of 50% (T-IC₅₀) and 90% (T-IC₉₀) for each porphyrin-generated singlet oxygen treatment (porphyrins concentrations) and their relative Standard Errors (SE).

Treatment	Time-IC ₅₀ [h]	Time-IC ₉₀ [h]
Dark control	8.12 ± 0.23	12.44 ± 0.57
Light control	7.34 ± 0.19	12.06 ± 0.68
1 μM	5.76 ± 0.10	10.51 ± 0.58
5 μM	3.13 ± 0.04	4.88 ± 0.14
10 μM	2.12 ± 0.02	2.81 ± 0.08
20 μM	1.62 ± 0.01	2.00 ± 0.04

Ideally, a good PS, should not cause toxicity in the dark (Detty et al., 2004; Mroz et al., 2007; Sharma et al., 2011). Both porphyrins used in this work (TMPyP: Figure 3.5 and TPPS₄: Figure 3.6), demonstrated that porphyrins-generated singlet oxygen

showed no toxicity *per se* (dark control) and did not cause bacterial luminescence signal reduction. In addition, no toxic effects of light irradiation alone were recorded in the light control.

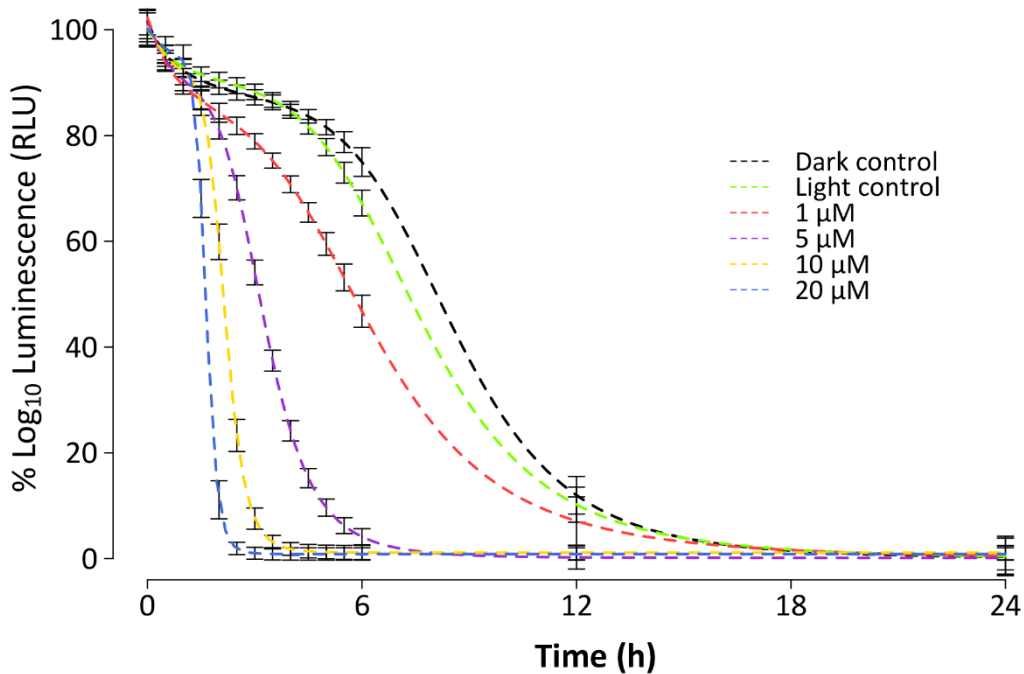


Figure 3.5: Time-course of photoinactivation of the luminescence signal of *V. campbellii* ISO7 using the tetra-cationic porphyrin (TMPyP). Error bars show model-based standard errors (n=3) (Ritz and Streibig, 2016).

The luminescence signal of *V. campbellii* ISO7 treated with the tetra-cationic porphyrin (TMPyP) showed a clear time-dependent dose-response of decreasing signal strength with increasing porphyrin concentration when exposed to light source (Figure 3.5, Table 3.1). The created model described dose- and time dependency of the treatments accurately (Cedergreen-Ritz-Streibig model; Lack-of-Fit-F-test: F-value=0.96, p =0.56, DF=60). Pairwise comparison between treatments required to reach a 50% (T-IC₅₀) of luminescent inhibition are presented in Table 3.2, respectively. The luminescence signal for the 1 μM porphyrin treatment declined to 50% ~1.4 and ~1.3 times more rapidly than seen for the dark and light controls, respectively (Table 3.2). In comparison, the luminescence signal of the 20 μM porphyrin treatment was reduced to 50% about 5 times more rapidly when compared to control groups. Both dose and time significantly affected the luminescence signal (Kruskal-Wallis $\chi^2= 45.21$, df = 5, p = 1.32 x 10⁻⁰⁸, $\chi^2=194.03$, Df =14, p < 2.2 x 10⁻¹⁶, respectively). Dunnett T3 test confirmed that samples treated with porphyrin concentrations of 5 μM or higher were

significantly different to the control groups Figure 3.7. However, while there was a small difference between luminescence curves for the lowest porphyrin concentration (1 μM) and the controls, this difference was not significant (Figure 3.7).

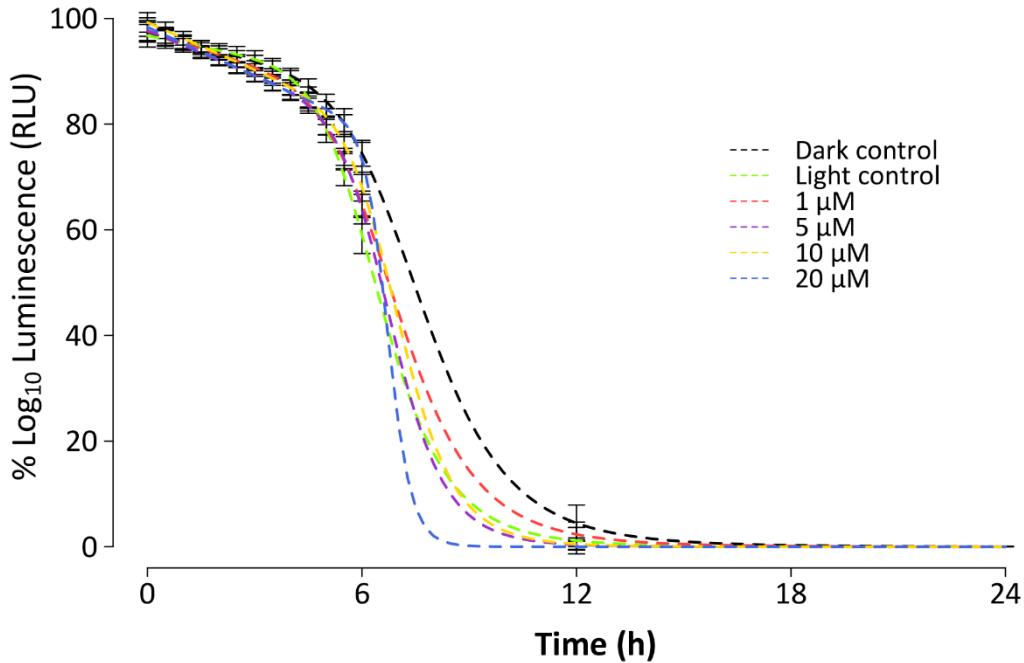


Figure 3.6: Time-course of photoinactivation of the luminescence signal of *V. campbellii* ISO7 using the tetra-anionic porphyrin (TPPS₄). Error bars show model-based standard errors (n=3) (Ritz and Strebig, 2016).

Treatments using the tetra-anionic porphyrin (TPPS₄) did not induce a dose-dependent luminescence decline (ANOVA: Df = 5, Sum² = 261, Mean² = 52.3, F-value = 0.053, Pr(>F) = 0.998) when exposed to light irradiation (Figure 3.6), and hence CFU counts and regrowth assays were not performed for these treatments. This result agreed with published data on antimicrobial activity of anionic photosensitisers against Gram-negative bacteria (Alves et al., 2009; Benov, 2015; Ergaieg and Seux, 2009; Gsponer et al., 2015; Jori and Coppellotti, 2007; Sperandio et al., 2013; Vatansever et al., 2013; Wikene et al., 2015).

The luminescence signal naturally declined in our assay after about 6 h, in both the light and dark controls (Figure 3.5). This was most likely due to nutrient and oxygen limitations (De Zwart and Slooff, 1983). This natural signal decline limits the usefulness of luminescence assays as a reporter tool for cytotoxicity assessments to shorter time frames; this is also reflected in the short incubation time (4.5 h) used in the study by Alves et al. (2011b). In order to increase the sensitivity and extend the treatment times

in our study, we included additional assays (absorbance measurements, CFU counts and regrowth assays) that could complement the rapid bioluminescence assay.

While the toxic effect of light irradiation alone and presence of porphyrins in the dark were investigated using reduction of luminescent signal in presence of TMPyP or TPPS₄, more detailed investigation was conducted using TMPyP and absorbance (570 nm) to detect bacterial biomass. As shown in Figure 3.8, absorbance of dark controls increased, reflecting growth. Porphyrin-treated bacterial suspensions exposed to light showed an initial small increase in absorbance indicative of initial growth, but this effect was inversely correlated with porphyrin concentration and not sustained over time (Figure 3.8). There were significant differences between the ABS₅₇₀ treatments (Kruskal-Wallis $\chi^2 = 107.62$, $df = 4$, $p < 2.2 \times 10^{-16}$) confirming a dose-effect but no time dependent effect (Kruskal-Wallis $\chi^2 = 22.646$, $df = 14$, $p = 0.07$). A post-hoc Dunnett T3 test showed no significant difference between dark control and lowest porphyrin concentrations (data not shown).

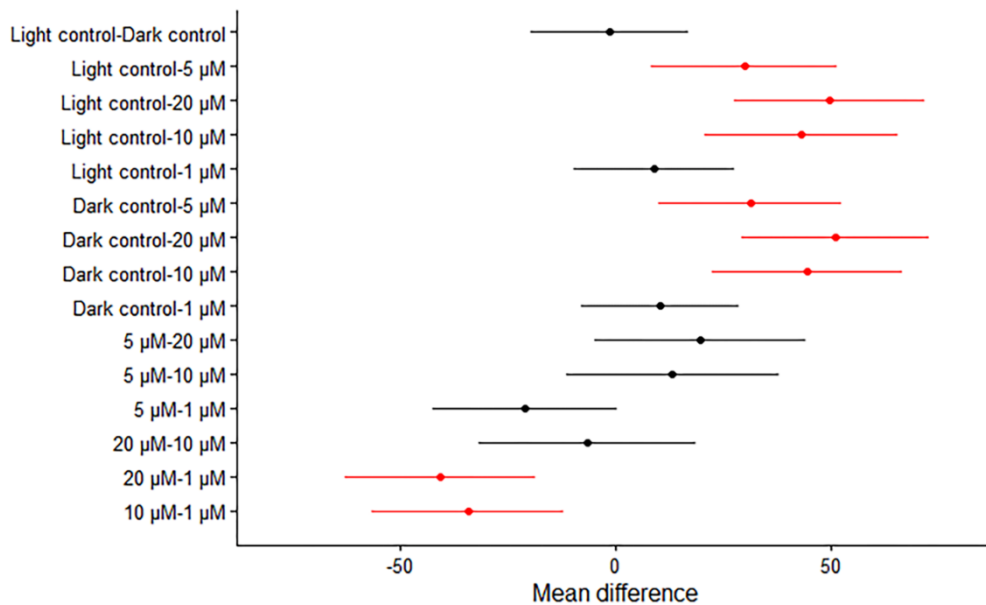


Figure 3.7: Dunnett T3 test result for the mean different %Log luminescence reduction between treatment groups. Point = mean difference, bars = confident interval, Red bars = significant difference, Black bars = no significant difference, $\alpha = 0.05$.

The CFU counts and 7-day regrowth assays demonstrated 100% lethality to *V. campbellii* ISO7 when exposed to 20 μM of the tetra-cationic porphyrin TMPyP and an irradiation of 223.8 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 5 h (e.g. no regrowth after 7 days), whereas 12 h, 12 h and 24 h treatment times were required to achieve a corresponding lethal effect

at TMPyP concentrations of 10 μM , 5 μM and 1 μM porphyrin, respectively (Figure 3.8).

Table 3.2: Pairwise comparison ($\alpha=0.05$) of time effect (T-IC₅₀) to examine relative potential (RP) of treatments. RP is calculated as ratio between T-IC₅₀ treatment 1 and 2 (Tr₁ against Tr₂). RP = 1, treatment has the same rate of luminescent signal decline rate. RP > 1, in Tr₁ the signal declined less rapidly than in Tr₂. RP <1, in Tr₁ the luminescent signal declines faster than in Tr₂.

IC ₅₀ comparisons	RP estimate	t-value	p-value
1 μM /10 μM	2.71 \pm 0.06	30.26	< 2.2 x 10 ⁻¹⁶
1 μM /20 μM	3.54 \pm 0.07	34.91	< 2.2 x 10 ⁻¹⁶
1 μM /5 μM	1.84 \pm 0.04	20.32	< 2.2 x 10 ⁻¹⁶
1 μM /Dark control	0.71 \pm 0.02	-12.08	< 2.2 x 10 ⁻¹⁶
1 μM /Light control	0.78 \pm 0.02	-9.26	< 2.2 x 10 ⁻¹⁶
10 μM /20 μM	1.31 \pm 0.02	17.13	< 2.2 x 10 ⁻¹⁶
10 μM /5 μM	0.68 \pm 0.01	-28.95	< 2.2 x 10 ⁻¹⁶
10 μM /Dark control	0.26 \pm 0.01	-93.28	< 2.2 x 10 ⁻¹⁶
10 μM /Light control	0.29 \pm 0.01	-88.27	< 2.2 x 10 ⁻¹⁶
20 μM /5 μM	0.52 \pm 0.01	-57.84	< 2.2 x 10 ⁻¹⁶
20 μM /Dark control	0.20 \pm 0.06	-132.79	< 2.2 x 10 ⁻¹⁶
20 μM /Light control	0.21 \pm 0.01	-127.25	< 2.2 x 10 ⁻¹⁶
5 μM /Dark control	0.38 \pm 0.01	-50.76	< 2.2 x 10 ⁻¹⁶
5 μM /Light control	0.43 \pm 0.01	-46.30	< 2.2 x 10 ⁻¹⁶
Dark control/Light control	1.11 \pm 0.04	2.47	0.012

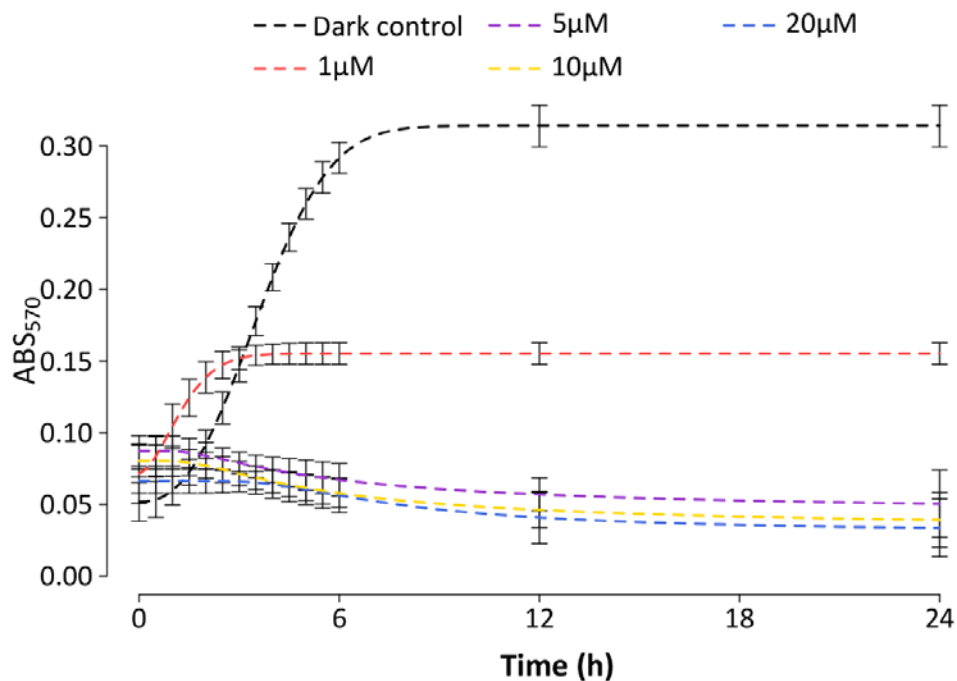


Figure 3.8: Time course of absorbance at 570 nm of *V. campbellii* ISO7 treated with the tetracationic porphyrin (TMPyP). Error bars show model-based standard errors (n=3)(Ritz and Strebig, 2016).

As expected, rapid regrowth occurred in the tested 24 h samples of the light and dark controls, supporting our hypothesis that the observed reduction in luminescence in these samples was due to limiting factors such as oxygen or nutrients. This highlights that the sensitivity of the luminescence assay to such limiting conditions needs to be considered in cytotoxicity assays. In addition, the results showed that CFU counts and the regrowth assays have their own limitations, as positive detection relies on chance, which is 10 cells·mL⁻¹ per plate or tube. For instance, with 20 μM porphyrin the luminescence signal reached its detection limit after 4 hours and no colonies were formed on any of the three agar plates, suggesting 100% lethality of the treatment. However, one of the three replicates in the regrowth assay showed turbidity (luminescent cells), suggesting that at least 1 cell was alive in the inoculum used for that tube, although contamination could not be 100% excluded.

Table 3.3: Regrowth assays (n=3) and CFU counts (n=3) performed when the luminescence signal reached the detection limit.

Porphyrin (μM)	Time (h)					Regrowth assay* ((CFUs·mL ⁻¹)) **
	4	5	6	12	24	
20	1(0)	0(0)	0(0)	0(0)	0(0)	
10		2(0)	1(0)	0(0)	0(0)	
5			3(8.8 x 10 ⁻¹)	0(0)	0(0)	
1				3(>300)	0(0)	
0					3(>300)	
Dark control					3(>300)	

* Number out of 3; * Colony forming units >300 CFUs·mL⁻¹ (upper detection limit) were considered as uncountable.

According to our assays, complete inhibition of bioluminescence activity was achieved for 10 and 20 μM porphyrin (TMPyP) irradiated at 223.8 μmol m⁻²s⁻¹ (corresponding to 1.179 mW cm⁻²) for 5 h, and complete lethality was achieved for the 20 μM porphyrin treatment under these conditions. In comparison, Alves et al. (2011b), demonstrated a complete inactivation of the natural luminescent *Vibrio fischeri* after ~4.5 h under 4 mW cm⁻² irradiation in the presence of 10, 20 and 50 μM of 5,10,15-tris(1-methylpyridinium-4-yl)-20-(pentafluorophenyl)porphyrin tri-iodide (Tri-Py⁺-Me-PF) porphyrin diluted in filtered (0.22 μm) aquaculture water. We note that we achieved similar inhibition of bioluminescence using four times lower

irradiation. It appears that the tetra-cationic porphyrin TMPyP generated more $^1\text{O}_2$ per photon absorbed (higher quantum yield) and may hence be more suitable for aquaculture purposes. $^1\text{O}_2$ quantum yield for Tri-Py⁺-Me-PF and TMPyP remain to be established under identical conditions to validate this hypothesis. In another study, the effect of 1 μM of the cationic porphyrins 5, 10, 15, 20-tetrakis(4-(N-(trimethyl) ammonio) phenyl)-21H, 23H-porphine tetrakis(p-toluenesulfonate) (TTMAPP) or TMPyP on the survival of *Escherichia coli* was determined by CFU counts. $32 \pm 21\%$ and $30 \pm 5\%$ reductions in growth were achieved after 10 min of treatment with 25 mW cm^{-2} irradiation using a 400 W halogen lamp, respectively (Komagoe et al., 2011). Our culture-based assays were not designed to detect such high cell numbers (30% survival would correspond to $3.3 \times 10^6 \text{ cells}\cdot\text{mL}^{-1}$), hence the results from the two studies are not directly comparable. To achieve the same level of irradiance for the TMPyP used here and in Komagoe et al. (2011), we need to multiply the treatment time of 10 min by 24. This corresponds to a treatment time of 4 h under our treatment conditions. We observed a reduction in bioluminescence of $\sim 37\%$ for the 1 μM TMPyP treatment which correlates well with the data of Komagoe et al. (2011). As evidenced, light intensity and exposure duration determine effectiveness of the treatments which needs to be considered in on-farm approaches for achieving 100% lethality to avoid activation of ROS defence mechanisms and the need of higher dosages. The results demonstrate the suitability of TMPyP for *in situ* treatment in aquaculture, whereas photobleaching avoids its build-up in water and animals, making it a suitable photosensitizer for environmentally friendly PACT treatment.

3.4 Summary

Photodynamic Antimicrobial Chemotherapy (PACT) has emerged as a promising method for pathogen eradication and control. PACT uses light excitation of non-toxic photosensitisers to produce singlet oxygen ($^1\text{O}_2$), which in turn damages and eradicates microbial cells. In the present study, a naturally luminescent *Vibrio campbellii* strain ISO7 (*V. campbellii* ISO7) was used as a model aquaculture pathogen to test the suitability of two porphyrin compounds, the tetra-cationic TMPyP and the tetra-anionic TPPS₄, for the treatment of aquaculture water. Initial photobleaching tests demonstrated natural degradation of both porphyrins after continuous

irradiation, making them suitable as 'self-destructive' photosensitizers for *in situ* treatment of aquaculture waters, as they do not accumulate in the water. In separate time-course experiments, the two photosensitisers were diluted in aquaculture water seeded with the indicator bacterium and samples were irradiated for 24 h using 150 W white LED light. Luminescence assays, growth and regrowth experiments demonstrated that the cytotoxicity of generated $^1\text{O}_2$ was both time- and dose-dependent, and confirmed that light or porphyrins alone were not toxic. Continuous irradiation in the presence of 20 μM cationic porphyrin for 5 h or 1 μM for 24 h achieved complete lethality of the indicator bacterium. Consistent with previous reports, the tested anionic porphyrin did not impact on the survival of the bacterium, causing only a slight decline in the luminescence signal. Although photobleaching indicated potential of using both porphyrins in aquaculture, the result obtained from the time-course experiment suggest that the TMPyP porphyrin is the better candidate and was therefore used in the following chapters.

4 Chapter 4: ⁴Sensitivity of Live Microalgal Aquaculture Feed to Photo Antimicrobial Chemotherapy

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4.1 Introduction

Marine microalgae are at the base of most marine food webs and are a key determinant for the primary productivity on the planet. Other than their ecological relevance, microalgae are increasingly becoming an important resource in today's economy. Rapid cell division and high specific nutritional values (i.e. fatty acid -, protein -, carbohydrate - and anti-oxidant contents) typical of microalgae are among the reasons for growing interest in commercial production (Natrah et al., 2014; Salvesen et al., 2000; Zhang et al., 2014a). Biomass of marine microalgae is produced for multiple purposes: high-energy food for human and animal consumption (Liu and Chen, 2016), environmental clean-up (Umamaheswari and Shanthakumar, 2016), cosmetic applications (Fernandes et al., 2015) and could potentially be used for biofuel production (Borowitzka and Moheimani, 2010; Islam et al., 2013; Liu and Chen, 2016; Pandey et al., 2014; Sing et al., 2013). Today, demand for microalgal biomass is primarily driven by use in the aquaculture industry, where it is used as live feed for a

⁴ This chapter will be submitted to "Journal of Applied Phycology".

wide range of organisms including molluscs, crustaceans and fish, including zooplankton enrichment (Salvesen et al., 2000; Zhang et al., 2014a).

Phytoplankton could act as vectors, transferring pathogenic bacteria to hatcheries or grow-out ponds when used as feed. Therefore, microalgal biomass may present a major threat with regards to biosecurity for the industry when produced and used as feed in aquaculture facilities. Bacteria can network with microalgae in a mutual or parasitic interaction (Natrah et al., 2014; Pintado et al., 2014; Unnithan et al., 2014). This interaction can have positive or negative effects on opportunistic bacteria, stimulating or inhibiting bacterial growth *via* algae exudate production (Natrah et al., 2014; Pintado et al., 2014; Unnithan et al., 2014). Generally, bacterial biomass increases when microalgae growth rates decrease (stationary phase) (Salvesen et al., 2000). In addition, algae are able to produce antibacterial substances that affect bacterial flora (Duff et al., 1966; Lam et al., 2008; Nagayama et al., 2002; Unnithan et al., 2014). However, antibacterial substances are more efficient against opportunistic bacteria in combination with increased competition for nutrients when microalgal cultures are in early stationary growth phase, as in this phase, both algal and bacterial biomass increase (Borowitzka, 1995). Surprisingly, opportunistic bacteria belonging to the genus *Vibrio* are not efficient competitors and less abundant in the stationary phase of algae cultures (Salvesen et al., 2000). *Vibrio* spp are, also, a group of bacteria that contain pathogenic species and highest bacterial densities occur when nutrients are available (Colwell, 1984).

In aquaculture, microalgae cultures are added as feed for animals at regular intervals which may result in a continuous bacterial inoculum into the animal culture system. Approximately 10^8 CFU of bacteria per algae supplement per litre of bivalve larval culture have been estimated to be introduced (Murchelano and Brown, 1969; Nicolas et al., 2004; Salvesen et al., 2000). While the recurrent introduction of bacterial flora might have positive effects on the bacterial community of larval system, the introduction of highly opportunistic bacteria in high nutrient environments, however, should be avoided (Salvesen et al., 1999). Pathogenic bacteria associated with microalgae feed in an aquaculture facility could potentially lead to disease outbreak which can have severe repercussion for the business (Natrah et al., 2014; Pintado et

al., 2014; Unnithan et al., 2014). For example, Gomez-Gil et al. (2002) reported that ideal growth conditions for *Vibrio alginolyticus*, a potential aquaculture animal pathogen, occur in cultures of the widely used microalga *Chaetoceros muelleri*. Salvesen et al. (2000) counted up to 10^3 CFU·ml⁻¹ of *Vibrio* bacteria in cultures of *Pavlova lutheri*. Thus, intensive aquaculture operations allocate large resources to controlling bacterial loads in microalgal cultures, with new technologies being continuously developed (Pintado et al., 2014). Creating and maintaining axenic microalgae cultures on a large scales is not only challenging and often unrealistic (Pintado et al., 2014), but carry the risk of slowed microalgae production (Cho et al., 2015; Watanabe et al., 2005), as phytohormones, macro- and micronutrients produced by bacteria increase algae growth rate (Bolch et al., 2011; Croft et al., 2005; Kazamia et al., 2012; Kim et al., 2014; Kuo and Lin, 2013; Ramanan et al., 2016; Teplitski and Rajamani, 2011). Hence new strategies are needed to minimise the presence of potential pathogenic bacteria and ideally create axenic “ready to feed” algae-inoculi in aquaculture.

Although a wide array of sterilization techniques has been developed specifically for the reduction of bacteria in aquaculture, each technology has its own limitations as discussed previously (1.5). The production of ROS, such as ¹O₂, during PACT is considered an innovative sterilization method within the aquaculture industry, as it creates a toxic environment for microorganisms, including pathogenic bacteria that lack specific ¹O₂ detoxification system (Maisch, 2015b). Generally, bacteria might express enzymes such as superoxide dismutase, catalase and peroxidase (ROS detoxification system) that increase cell survival when exposed to sublethal PS dosages. These ROS-detoxifying enzymes are, however, less efficient in combating ROS-damage afflicted externally (i.e. cell wall and cell membrane) (Maisch, 2015b). Therefore, bacterial cells are usually unable to tolerate ROS and ¹O₂ build-up in the environment. Furthermore, superoxide dismutase, catalase and peroxidase are not able to quench ¹O₂ (Wainwright and Crossley, 2004) and are degraded by ¹O₂ (Kim et al., 2001). Photosynthetic microorganisms, on the other hand, have specific defence mechanisms to protect themselves from possible damage by internal ROS and ¹O₂. The algal antioxidant defence system is composed of both antioxidant enzymes such as

superoxide dismutase, catalase, guaiacol peroxidase, enzymes of the ascorbate-glutathione cycle ascorbate peroxidase (mono-dehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase) and non-enzymatic components such as cellular oxido-reduction buffers (ascorbate and glutathione) and compounds (tocopherols, phenols and carotenoids), with the latter being capable of quenching $^1\text{O}_2$, while the enzymes fulfil the same ROS-detoxification function as in animal -, plant - and bacterial cells (Glaeser et al., 2011; Noctor and Foyer, 1998; Sharma et al., 2012; Vatansever et al., 2013). Few studies have been conducted to confirm the higher tolerance of photosynthetic organisms to ROS and $^1\text{O}_2$ build-up. For instance, Drabkova et al. (2007) showed that microalgae and cyanobacteria are both sensitive to singlet oxygen. Furthermore, Pohl et al. (2015) showed that green algae are sensitive to cationic PS but not to anionic ones. However, the current paucity of evidence on microalgal resistance to PACT makes it difficult to estimate how effective these techniques could be for aquaculture microalgal feed sterilisation purposes.

The aims of the present study were 1) to test the toxicity of $^1\text{O}_2$ generated by the cationic PS TMPyP during PACT (3.2.1) towards the commonly used microalgal aquaculture species: *Tisochrysis lutea* (T-ISO) (NQAIF001), *Nannochloropsis oculata* (NQAIF283), *Tetraselmis chui* (NQAIF289), *Chaetoceros muelleri* (CS-176) and *Picochlorum atomus* (NQAIF284); and 2) select one or more PACT-resilient microalgal species to test in mixed culture with the model bacterium *V. campbellii* ISO7 (2.3.1). This study represents the first documentation of porphyrin-generated $^1\text{O}_2$ toxicity on aquaculture phytoplankton species.

4.2 Materials and Methods

4.2.1 Toxicity test

4.2.1.1 Algae strain and growth condition

Marine microalgae used in this experiment were obtained from the North Queensland Algae Identification Facility (NQAIF, James Cook University; Table 4.1) [*Tisochrysis lutea* (T-iso) (NQAIF001), *Nannochloropsis oculata* (NQAIF283), *Tetraselmis chui* (NQAIF289) and *Picochlorum atomus* (NQAIF284)] and from AIMS (Australian

Institute of Marine Science, Townsville, Australia) [*Chaetoceros muelleri* (CS176)]. Cell sizes of these microalgal species varied and are reported in Table 4.1.

Microalgae species were selected based on their hatchery application in aquaculture to 1) feed animal larvae (Brown and Blackburn, 2013; FAO, 2007), 2) their cell wall composition and 3) their fatty acid profiles and lipid content.

Microalgae were either cultured in modified L1 liquid medium (Guillard and Hargraves, 1993) or f/2 medium (Guillard, 1975). The medium was sterile filtered rather than autoclaved to avoid precipitation and high background noise in the subsequent flow cytometer analysis. In the modified protocol, seawater was filtered through a 0.22 µm Durapore membrane filter (Millipore, North Ryde, Australia) and all culture media components were filtered through a 0.22 µm syringe filter (Minisart high-flow membrane filter, Sartorius Stedin Biotech, Göttingen, Germany).

Table 4.1: Cell size and culture collection accession numbers of microalgal species used. *Previously described as *Isochrysis affinis galbana* (Bendif et al., 2013).

Specie	Cell size (µm)	Accession number	Reference
<i>P. atomus</i>	2-3	NQ284	Butcher (1952); Henley et al. (2004)
<i>N. oculata</i>	2-4	NQ283	Beacham et al. (2014); Hibberd (1981); Yamamoto et al. (2003)
<i>T. lutea (T-iso)*</i>	4.5–7.5 long and 3–6 wide	NQ001	Bendif et al. (2013)
<i>C. muelleri</i>	8 long and 5 wide	CS176	Martinez-Fernandez et al. (2006)
<i>Te. chui</i>	12-16 long and 7-10 wide	NQ289	Butcher (1959); Hori et al. (1986)

4.2.1.2 Photosensitizer

The PS used in this work was the cationic porphyrin TMPyP described previously (3.2.1). Before the experiment, a stock solution was prepared by diluting the PS in 100% dimethyl sulfoxide (DMSO) to a concentration of 10 mM (stock solution), which was stored in 2 mL Eppendorf tubes containing 1.5 mL of stock solution at 3–4 °C. Aluminium foil was used to shield the PS from any light exposure. At the start of experiments, the PS stock solution was diluted in L1 or f/2 medium (data not shown as similar to L1 medium) at the concentration described in 4.2.1.3. Figure 4.1 presents the molecular structure and full absorbance spectra (350 and 750 nm) of TMPyP diluted in L1 medium at concentrations between 0 and 20 µM.

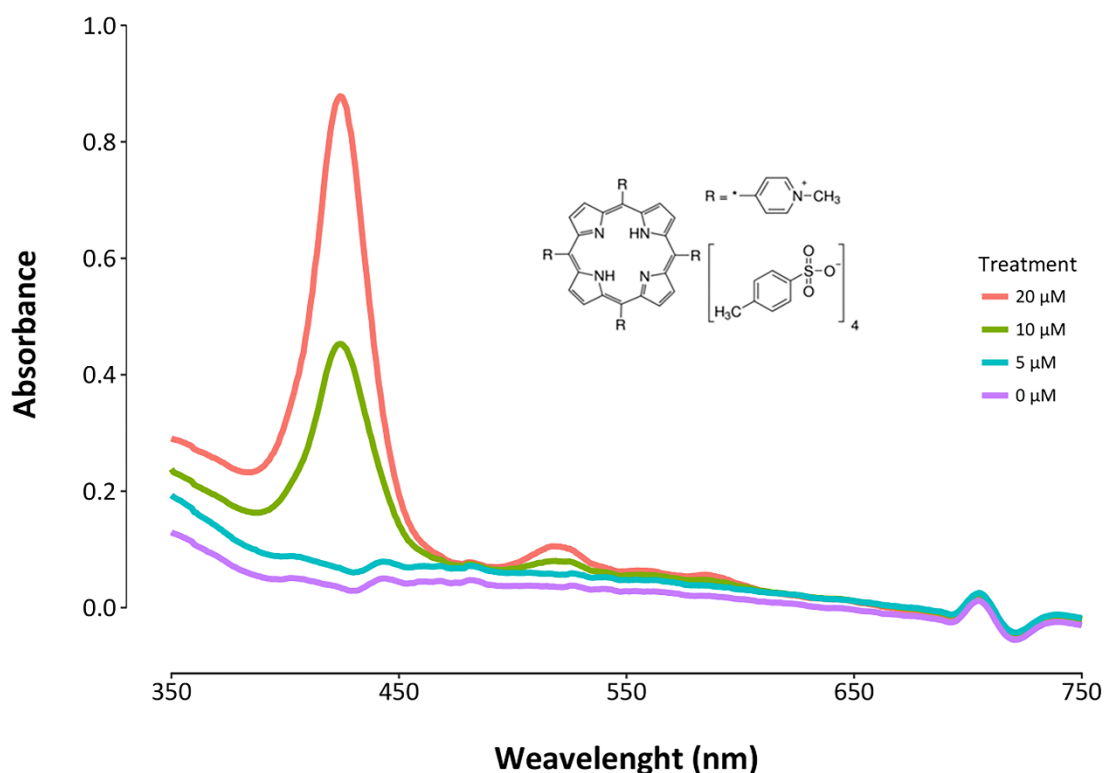


Figure 4.1: Molecular structure and absorbance spectra (350-750 nm) of TMPyP porphyrins diluted to final concentrations of 20, 10, 5 and 0 μM in L1 medium.

4.2.1.3 *Microalgae viability evaluation*

Microalgae viability was evaluated using a flow cytometer (InCyte, benchtop Merk Millipore, Bayswater, VIC, Australia) and the dye Propidium Iodide (PI). The advantage of using PI is that it does not pass through cell membranes and can therefore bind nucleic acids only when cells are damaged (dead) (Berney et al., 2007; Darzynkiewicz et al., 1994). Propidium Iodide (Aldrich, Australia) was diluted in Phosphate Buffer Saline (PBS) at a concentration of 3.5 mg PI in 1 mL PBS and stored at 4°C in the dark for the entire experiment. The protocol was adjusted for each microalgal species by modifying the counting box based on cell size [forward scatter (FSC), chlorophyll auto red fluorescence (RF) and/or side scatter (SSC) (Veldhuis and Kraay, 2000)]. To avoid issues with overlapping RF signals from algae auto-fluorescence and PI (Olson and Chisholm, 1986; Van Bleijswijk and Veldhuis, 1995), the yellow channel (yellow fluorescence [YF]) was used to detect the PI signal (details on PI fluorescence excitation and emissions can be found on www.invitrogen.com). Before each experiment and for each microalgal species, the protocol was optimised as follows: 1) one aliquot (4 mL) of fresh microalgal culture was split into 2 equal volume

samples before each experiment, one of which was untreated (live cells) and the other was placed in boiling water for 20-30 min (dead cells); 2) cells were selected using SSC~FFC or RF~FFC plots and gated in the final plot RF~YF where live and dead counting box were created (Figure 4.2).

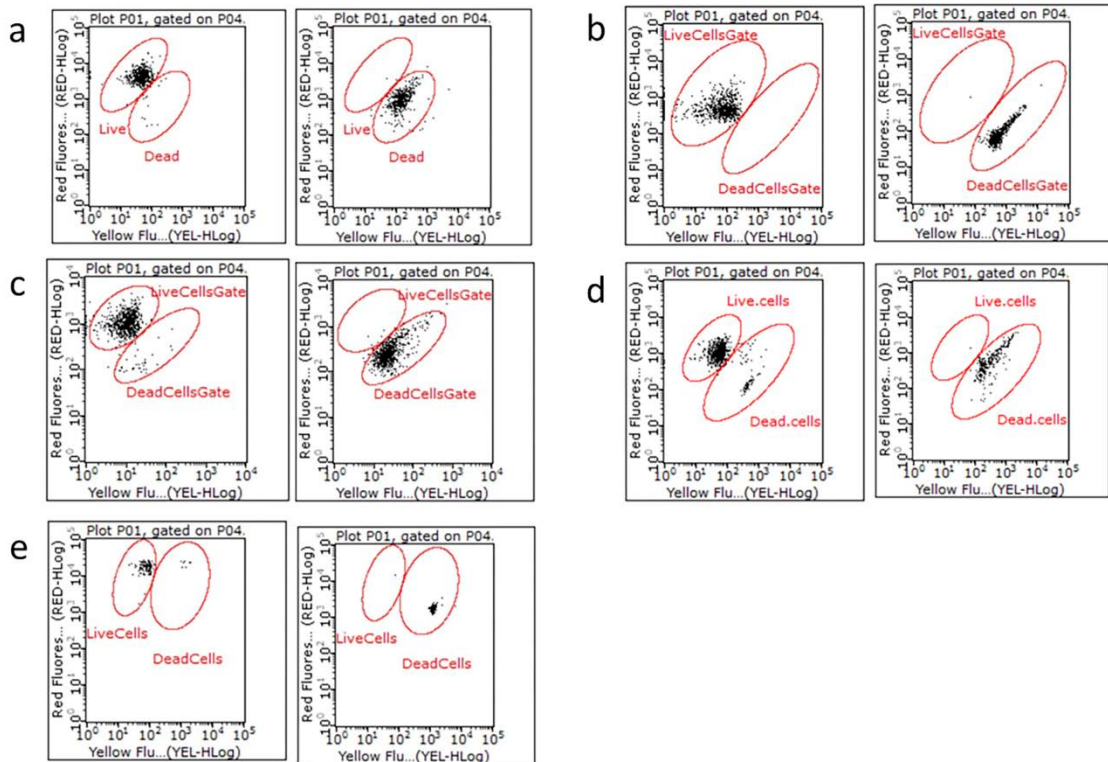


Figure 4.2: Flow cytometer determination of live and dead (by heating) cell determination of *C. muelleri*^a, *T. lutea*^b, *N. oculata*^c, *P. atomus*^d and *Te. chui*^e at the start of the experiment using the dye PI.

4.2.1.4 *TMPyP-generated photoinactivation of microalgae used in aquaculture*

Considering the difference in cell size of the five microalgae species used, we normalised culture inoculation for each species using dry weight rather than cell number. Table 4.2 shows the equations used to transform cell number to dry weight ($\text{g}\cdot\text{L}^{-1}$) for each organism. Microalgal culture were maintained in L1 medium, with the exception of *C. muelleri* cultures, which was maintained in f/2 medium to a final dry weight concentration of $\sim 0.04 \text{ g}\cdot\text{L}^{-1}$. Final concentrations of the porphyrin TMPyP were 50, 20, 10, 5 and 1 μM with final DMSO concentration kept constant for all treatments (0.5%). Preliminary experiment using *T. lutea*, demonstrated that DMSO showed toxicity at concentration higher than 1% (data not shown). Possible solvent toxicity

effects were monitored for all microalgae species during using controls with DMSO (0.5%) but no porphyrin (light control; Figure 4.3).

Samples (2.5 mL) were placed in 12 well tissue culture plates (product # 353043, Beckton Dickinson, New Jersey, USA) (n=3 independent samples). For both samples and control, 3 independent samples were used as outlined in Figure 4.3. The light control (0 μM) was void of TMPyP, but contained 0.5% DMSO to monitor potential solvent toxicity and produced baseline response data for the microalgae. Dark controls received the highest porphyrin concentration (50 μM) and no addition of porphyrins (0 μM) and were wrapped in aluminium foil for protection from light. They were used to assess the toxicity of the PS (50 μM) and to test if the absence of light had negative effect of the microalgae population (0 μM).

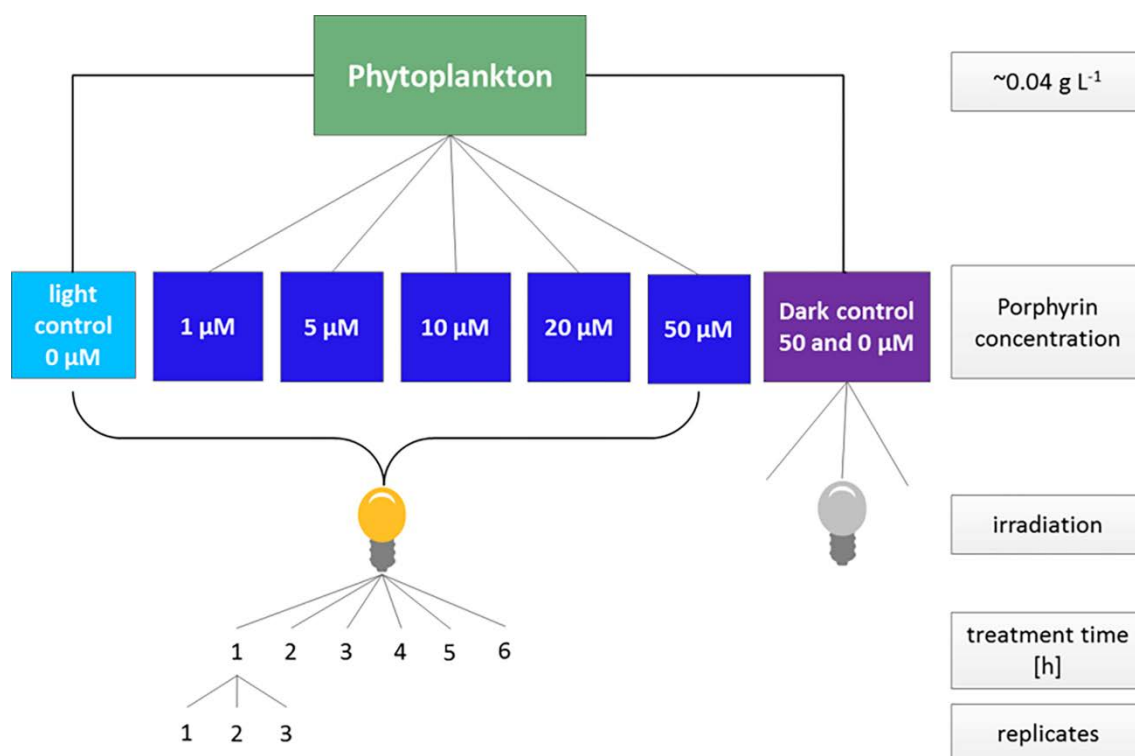


Figure 4.3: Schematic of the experimental design used in dose-response and time-course experiments.

Samples were incubated for 20 min in the dark to promote porphyrin-binding to the cells and were subsequently exposed to continuous light as reported in 3.2.3 for 6 h. Well-plates containing samples were placed on an orbital shaker as previously described in 3.2.3.

Unless stated otherwise (Table 4.2), dry weight calibration curves were obtained by diluting fresh cultures of each microalgal species (n=3) in 20% dilution steps using L1 or f/2 medium. 100% cultures received no dilution and media blanks (no culture addition) served as 0% calibration standards. Each replicate and dilution were filtered onto glass fibre filters (previously labelled, dried at 100 °C overnight, followed by pre-ashing at 500 °C overnight and weighing after cooling to room temperature in a desiccator).

Table 4.2: Regression equations for calculating dry weight from cell number (cells mL⁻¹) and or transmittance. DW= Dry weight, Cell#= cell number (cells mL⁻¹), T= Transmittance.

Specie	Equation	Reference
<i>C. muelleri</i>	$DW = ((6 \times 10^{-08} \times \text{Cell \#}) + 0.0039)$	Dry weight determination created in this study*
<i>P. atomus</i>	$DW = ((-0.0051 \times T) - 0.5015)$ $\text{Cell \#} = ((-2 \times 10^{08} \times T) + 2 \times 10^{10})$	Indirect methods for culture growth determination as described by von Alvensleben et al. (2013)
<i>T. lutea</i>	$DW = ((6.91 \times 10^{-08} \times \text{Cell \#}) + 0.033)$	Provided by Dr. Roger Huerlimann as part of his research in our laboratory (unpublished)
<i>Te. chui</i>	$DW = ((6.58 \times 10^{-07} \times \text{Cell \#}) + 0.0056)$	Dry weight determination created in this study*
<i>N. oculata</i>	$DW = ((6.26 \times 10^{-09} \times \text{Cell \#}) + 0.0015)$	Provided by Dr. Roger Huerlimann as part of his research in our laboratory (unpublished)

An aliquot (200 µL) was used to determine cell number using the flow cytometer (see 4.2.1.3 for details). After each filtration, filters were washed with ammonium formate (0.5 M, pH 8.0, adjusted with 1M NaOH) at the rate of 2:1 (Volume of culture filtered: ammonium formate). Each filter was placed in a drying oven (model FD 23, Tuttlingen, Germany) for 24 h at 100 °C. After 24 h, the weight of each filter was recorded (in triplicate and after cooling to room temperature in a desiccator) and the average weight was subtracted from the average of the filter weight before the filtration process and divided by the volume (mL) of algae filtered.

4.2.2 Simulation of microalgal culture contamination with model bacterium *V. campbellii* ISO7

The model bacterium used was the natural luminescent *Vibrio campbellii* ISO7 that showed virulency against *P. monodon* when injected (see section 2.3 for detail).

The bacterium was streak-plated on LA from cryopreserved stocks and incubated at 28°C for 24 h. After this time, one colony was resuspended in LM (30 mL) and grown as described in 3.2.2.

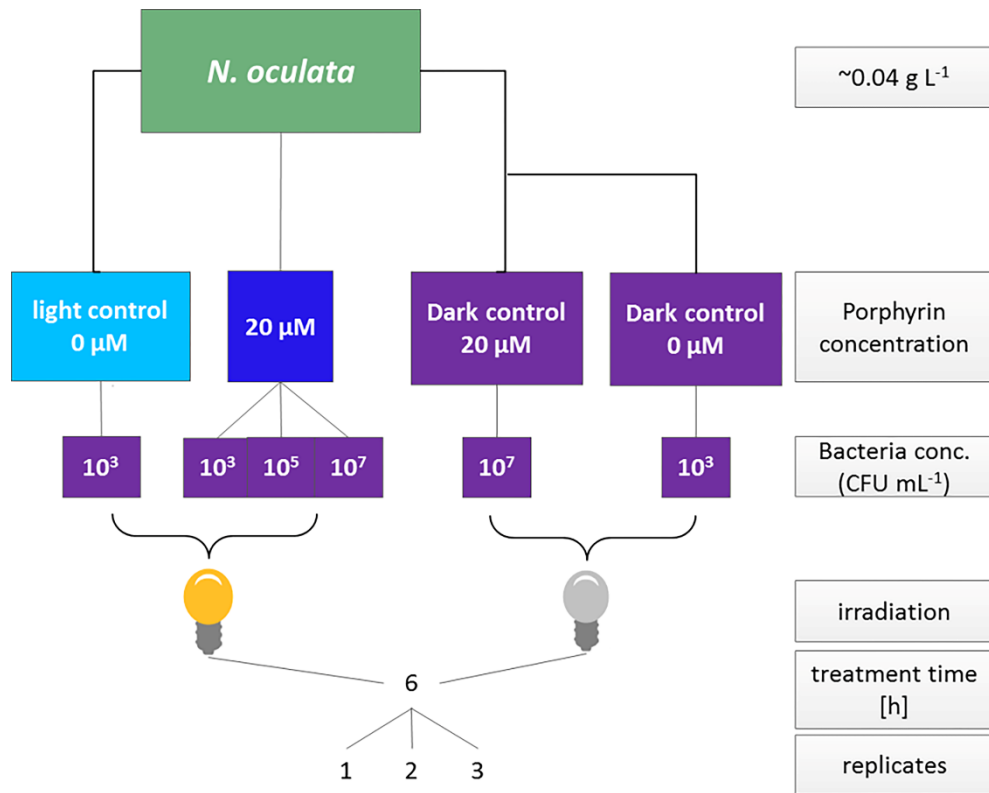


Figure 4.4: Experimental design of the simulation algae culture contamination.

This experiment used the PACT-resilient microalga *N. oculata* as a model and growth conditions were as reported in 4.2.1.1. The experimental design is presented in Figure 4.1. A fresh culture of *N. oculata* was diluted to a final concentration of $\sim 0.04 \text{ g L}^{-1}$ in L1 medium in the presence of 20 μM of TMPyP, except for the light control and the 0 μM dark control, and inoculated with different final concentrations of *V. campbellii* ISO7 (approximately 10^3 , 10^5 and 10^7 CFU mL^{-1}). Samples (2.5 mL) were added to a 12-well tissue culture plate (product # 353043, Beckton Dickinson, New Jersey, USA) ($n=3$ independent samples) and exposed to light irradiation as described in 4.2.1.1. At the start and end of the experiment (time 0 and 6 h), samples were taken for CFUs count and species-specific most probable number (MPN; 1.5 mL) determination. The number of CFUs on LA was determined by spread plating (0.1 mL, $n=3$) aiming to detect the luminescent signal from the bacterium. While a MPN-multiplex PCR strategy was used to estimate the number of *V. campbellii* ISO7 in mixed

cultures with *N. oculata* before and after PACT treatment. Each sample (1.5 mL) was centrifuged at 10000 rpm (rotor Eppendorf F45-30-11) for 15 min (Eppendorf 5810 R, Thermo Fisher Scientific Ltd, Australia) and washed with sterile L1 medium, followed by resuspension in 5 mL of Peptone Salt Solution (3% NaCl, 0.1% bacteriological peptone) and a final 1:10 mL diluted in Alkaline Peptone Water (mAPW; 1% bacteriological peptone, 3% NaCl). The DNA was extracted and amplified as previously reported (see 2.2.6).

4.2.3 Statistics analysis

Statistical analysis and graphs are presented in details in section A4. All analyses were performed using R-studio (v. 0.99.896) (RStudio Team, 2015), setting the significance level α to 0.05 and testing for normality and equal variance (*Rcdrm* package, v.2.3), using the Shapiro-Wilk test and the Levene's test, respectively (Fox, 2005, 2007).

For each microalgal species, treatment (porphyrin concentration) and time effects was investigated using either ANOVA (Chambers et al., 1992) or Kruskal-Wallis's test (Hollander and Wolfe, 1973; R Core Team, 2015) when ANOVA assumption were violated (*stats* package v.3.2.5). A pairwise post-hoc analysis (*PMCMR* package, V. 4.1) was used to investigate possible differences between time points (i.e. start and end of the experiment) or treatments (i.e. comparing control groups to treatment groups), specifically Tukey Honestly Significant Differences (Rupert G, 1981) was used for ANOVA-based statistics and a Dunn rank test after Kruskal-Wallis's statistical analysis (Dunn, 1964; Pohlert, 2014).

Dose-response analysis (*drc* package v.3.0.1) was conducted to identify which of the microalgal species was more sensitive to PACT treatment after 6 h of irradiation. Therefore, at the end of the experiment (time 6 h), the live cell proportion for each phytoplankton population was modelled against porphyrin concentration (0, 1, 5, 10, 20 and 50 μM). For each phytoplankton sample, Akaike's Information Criterion (AIC) was used to select the best fitting model over all possible candidates. The created models were used to identify the Inhibitory Concentration of 50% (IC_{50}) for the microalgal population after 6 h of irradiation (6-h IC_{50}) and establishing the relative

potency (RP) “interpreted as a measure for quantifying the strength” of one microalgal species over another (Ritz et al., 2015).

4.3 Results and discussion

4.3.1 Toxicity test

In general, porphyrin treatment (50 μM) in the absence of light and dark-exposure in the absence of the porphyrin TMPyP had no significant effect ($p > 0.05$; Figure A4.3) on population size (live, dead cells) of any of the five microalgae tested (Figure 4.5), proving that the porphyrin itself was not cytotoxic over the 6 h exposure and that the dark condition itself did not result in death of the microalgae.

Furthermore, the light source used did not photodestroy any of the microalgal species, as both control samples without porphyrin (0 μM) in the light and in the dark showed no significant difference ($p > 0.05$; Figure A4.3).

Table 4.3: ANOVA and Kruskal-Wallis rank sum table for detecting possible treatment effect.

Species	Df	χ^2/Sum^2	Mean ²	F-value	p-value/ Pr(>F)
<i>N. oculata</i>	7	73.2	10.46	1.248	0.28
<i>P. atomus</i>	7	95.87			$< 2.2 \times 10^{-16}$
<i>C. muelleri</i>	7	93.56			$< 2.2 \times 10^{-16}$
<i>Te. chui</i>	7	114.88			$< 2.2 \times 10^{-16}$
<i>T. lutea</i>	7	76.52			7.10×10^{-14}

In contrast, TMPyP concentration and exposure time affected four of the five microalgae tested (Figure A4.3). Only *N. oculata* showed no significant effect on population size at any of the TMPyP concentration over the entire time series ($p > 0.05$; Figure 4.5). A slight time effect at 6 h was detected by a small decrease in population size (Figure 4.5; Table 4.3 and Table 4.4), but this was not statistically significant (Figure A4.3).

Unlike *N. oculata*, *P. atomus*, *C. muelleri*, *Te. chui* and *T. lutea* generally showed high mortality when exposed to light in the presence of the cationic porphyrin TMPyP (Figure 4.5). Treatment responses together with all controls demonstrate that photooxidative damage was driven by TMPyP-generated $^1\text{O}_2$ (Figure A4.3 and Figure A4.4). Time and porphyrin dosage significantly decreased live cell density (time 0 and 6 h, $p < 0.05$, Figure A4.3), which were significantly lower than in controls ($p < 0.05$,

Figure 4.5, Figure A4.3). *Tisochrysis lutea* and *Te. chui* showed no significant differences to control groups ($p > 0.05$; Figure A4.4) at 1 μM TMPyP, demonstrating their ability to cope with low amounts of PACT-produced $^1\text{O}_2$.

Table 4.4: ANOVA and Kruskal-Wallis rank sum table for detecting possible time effect. Samples were divided into 1) TMPyP (1, 5, 10, 20 and 50 μM) + irradiation and 2) controls (light and dark controls).

Species	Samples	Df	χ^2/Sum^2	Mean ²	F-value	p-value/ Pr(>F)
<i>N. oculata</i>	TMPyP irradiated	6	429	71.50	22.96	$< 2.2 \times 10^{-16}$
	Controls	6	465	77.50	25.76	1.9×10^{-14}
<i>P. atomus</i>	TMPyP irradiated	6	69.488			5.2×10^{-13}
	Controls	6	41.009			2.9×10^{-7}
<i>C. muelleri</i>	TMPyP irradiated	6	76.026			2.4×10^{-14}
	Controls	6	4.7497			5.8×10^{-1}
<i>Te. chui</i>	TMPyP irradiated	6	22.093			1.2×10^{-3}
	Controls	6	6.4924			3.7×10^{-1}
<i>T. lutea</i>	TMPyP irradiated	5	41.839			6.4×10^{-8}
	Controls	5	4.0298			5.5×10^{-1}

Dose-response analyses after 6 h of continuous irradiations showed a species-specific response to porphyrin concentration (0, 1, 5, 10, 20 and 50 μM ; Figure 4.6). After 6-h of continues irradiation sensitivity to TMPyP-produced $^1\text{O}_2$ decreased in the following order; *P. atomus* \geq *C. muelleri* $>$ *Te. chui* $>$ *T. lutea* $>$ *N. oculata* (no effect), making the chlorophytes and bacillariophyte the most sensitive species. This was confirmed by pairwise species comparison using relative potency calculations based on the ratio of 6 h-IC₅₀ concentrations (Table 4.6). Furthermore, all porphyrin treatments for these microalgal species were significantly different to light controls (0 μM ; $p < 0.05$), supporting the conclusion that these microalgae are highly sensitivity to TMPyP-produced $^1\text{O}_2$ (Figure 4.6). *Tetraselmis chui* and *T. lutea* where intermediate: these species did not show mortality when incubated with 1 and 5 μM of TMPyP but populations exposed to higher TMPyP concentrations were significantly different from controls ($p < 0.05$) (Table 4.5: 6 h-IC₅₀=~4; Table 4.6: RP $>$ 1).

Based on results, TMPyP-generated $^1\text{O}_2$ showed species-specific toxicity against microalgal cells. For hatcheries, TMPyP-based PACT sterilization of the investigated microalgal feeds is only suitable for *N. oculata*. Lower dosages and shortened time

frames could be used for the other species, provided that time and dosage used would eradicate bacterial pathogens.

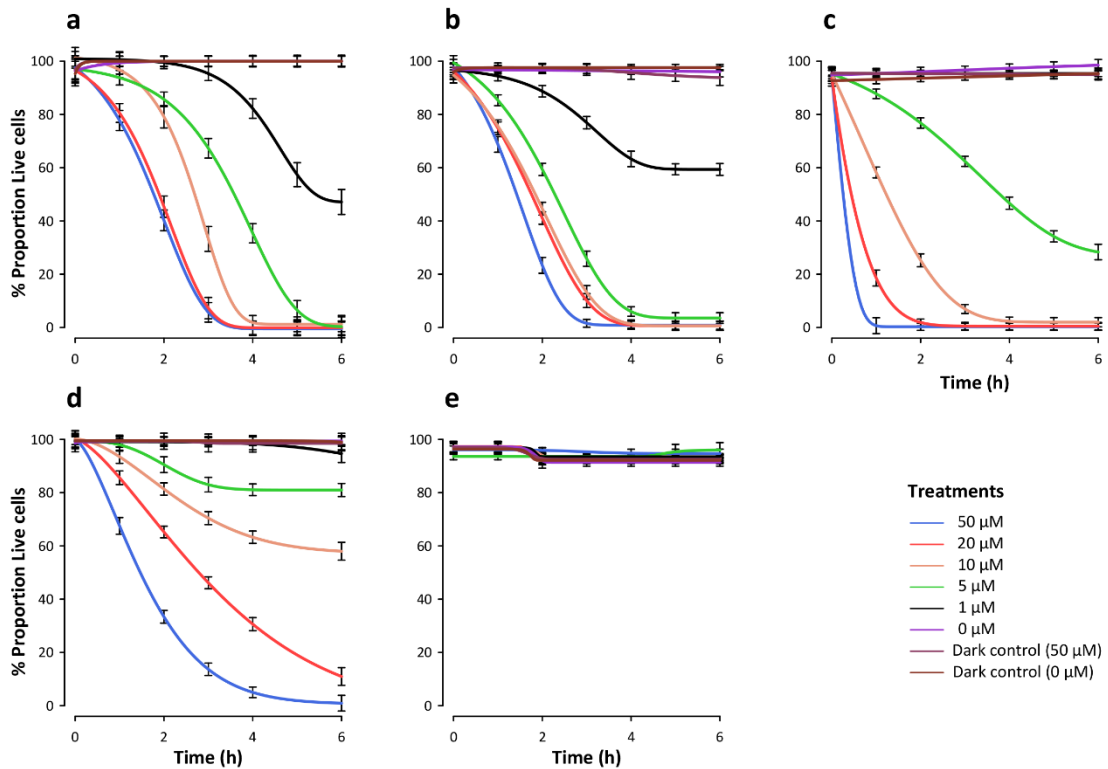


Figure 4.5: Effect of exposure time – and TMPyP concentration on population size (% of Live cells) of *P. atomus*^a, *C. muelleri*^b, *Te. chuii*^c, *T. lutea*^d and *N. oculata*^e. For each species, the best fitting model of all possible candidates was selected based on AIC values.

Except for *N. oculata*, the sensitivity of the other four microalgal species to TMPyP-produced ¹O₂ was surprising and not consistent with our understanding of microalgal ROS and ¹O₂ detoxification systems (Glaeser et al., 2011). Studies by Pohl et al. (2015) suggest a potential explanation for these unexpected results. Singlet oxygen produced by cationic porphyrins have been shown to degrade algal cell walls (Pohl et al., 2015). Results obtained in this study are consistent with published data, as no cell death occurred in the dark controls at 50 μM of the porphyrin TMPyP for any of the species. The observed species-specific sensitivity to ¹O₂ produced by the cationic porphyrin TMPyP can be explained by differences in the biochemical nature and organization of their cell coverings (Table 4.7).

Nannochloropsis oculata is a member of the Eustigmatophyceae and all representatives of the genus are characterized by a rigid bilayered cell wall, consisting of an inner cellulose and outer hydrophobic algaenan layer (Table 4.7) (Geldin et al.,

1999; Scholz et al., 2014). Our data suggest that the hydrophobic straight hydrocarbons chain that comprise the outer layer of the *Nannochloropsis* cell wall would yield the organism resilient to oxidation by the cationic porphyrin TMPyP-produced $^1\text{O}_2$.

Table 4.5: Inhibitory concentration of 50% of population size after 6 h of irradiation (6 h-IC₅₀ ± SE) for each microalgal species in a dose-response model. *N. oculata* returned infinite values and was not included.

Species	6 h-IC ₅₀
<i>P. atomus</i>	1.02 ± 0.06
<i>C. muelleri</i>	1.27 ± 0.07
<i>Te. chui</i>	4.04 ± 0.39
<i>T. lutea</i>	11.21 ± 0.28

Picochlorum atomus (syn. *Nannochloris atomus*) is a unicellular coccoid green alga belonging to the Trebuxiophyceae. The cell walls of the Trebuxiophyceae typically consist of cellulose, algaenan, and structural proteins (Table 4.7), whose disulphide linkages require reduction to weaken the cell wall (Porra, 2011). Since the cell wall of *Picochlorum* is thin (Table 4.7), it is possible that oxidation of structurally important proteins could allow TMPyP-produced $^1\text{O}_2$ to oxidise plasma membrane constituents, e.g. fatty acids, leading to the observed highest vulnerability to PACT. The frustule of diatoms is composed of three layers, an organic plasma membrane-associated layer (diatopetum), a mineralized silicified wall, which contains organic matter and the outmost cell wall-bound exopolysaccharide (EPS) layer (Gügi et al., 2015). *Chaetoceros muelleri* has been described as weakly silicified (hyaline), bearing a long seta at each corner of the cell, which has spirally arranged pores (puncta) with no opening at the end (Lemmermann, 1898; Reinke, 1984). The amount and monosaccharide composition of extracellular carbohydrates is influenced by pH (Thornton, 2009) and environmental conditions. In general, a glucuronomannan, i.e. blocks of 3-linked mannans substituted with glucuronic acid or sulphate groups in position 2 of the main chain are assumed common for diatoms (Gügi et al., 2015). To our knowledge, the frustule-associated polysaccharide composition and that of the EPS has not been characterized for *Chaetoceros muelleri*, but based on information obtained for several *Chaetoceros* species (Table 4.7), the cationic porphyrin is highly likely to complex tightly with frustule components, generating $^1\text{O}_2$ in very close

proximity to the cell, potentially destabilizing the thin and fragile frustule and plasma membrane phospholipids of the organism.

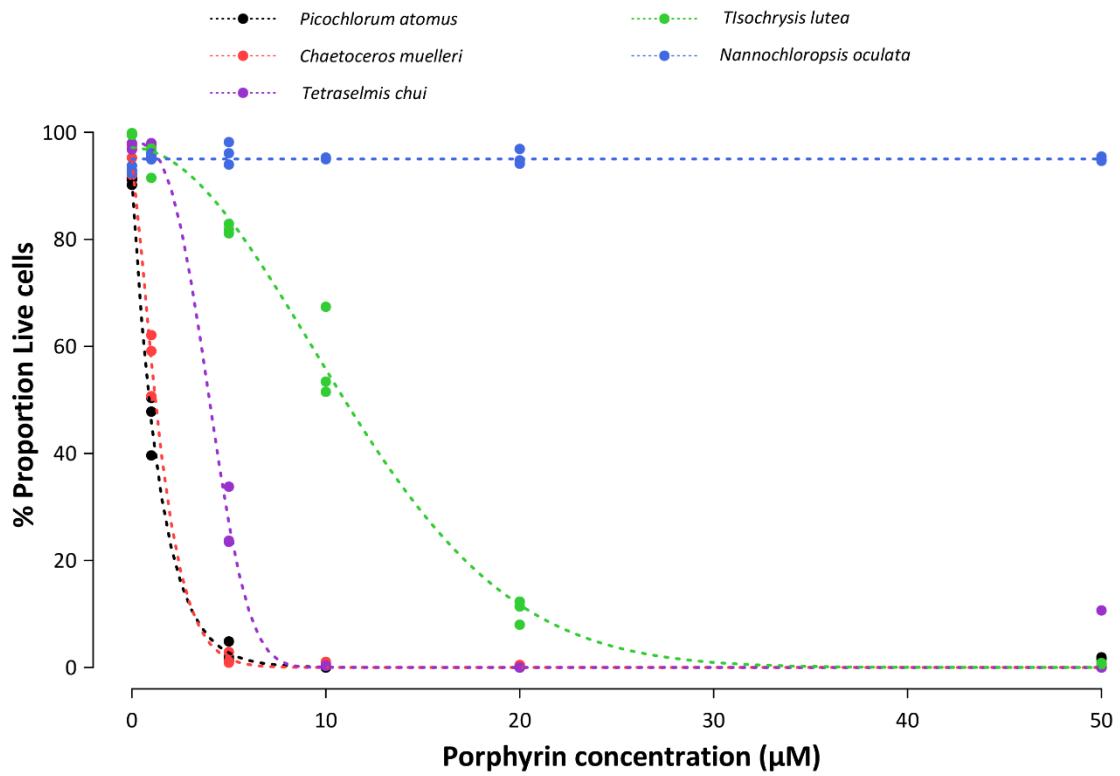


Figure 4.6: Dose-response curve of *N. oculata*, *P. atomus*, *C. muelleri*, *Te. chui* and *T. lutea* at time 6h. Model obtained using R-studio (version 0.99.01) and “drc” package.

As a prasinophyte, the theca of *Tetraselmis chui* is characterized by dominance of 2-keto sugar acids over neutral sugars (Table 4.7). Our culture pH (~8-9) would have induced a high density of negative surface charge on the *Te. chui* theca, enabling complexation of cationic porphyrin at higher concentration. This would have led to $^1\text{O}_2$ production in close proximity to the plasma membrane, explaining the observed sensitivity of this organism to TMPyP-generated $^1\text{O}_2$ of the organism studied.

The cell surface of the haptophyte *Isochrysis aff. galbana* (T-iso, Tahitian isolate), recently reclassified as *Tisochrysis lutea* (Bendif et al., 2013), is covered with thin non-mineralised organic scales bound together by acidic polysaccharides which are also thought to connect the cellulose microfibrils (Table 4.7). We hypothesise that the cationic porphyrin TMPyP could bind to these acidic polysaccharides destabilizing the organization of the cellulosic microfibrils. Singlet oxygen generation could then lead to oxidation of the underlying plasma membrane. Given the dense nature of these

organic scales, it is likely that the acidic polysaccharides are not easily accessible to TMPyP, which explains the lower sensitivity to TMPyP-generated $^1\text{O}_2$ and the longer time frames required for reduction in population size to occur.

Table 4.6: Relative potency (RP) calculated as the ratio of 6 h-IC₅₀ between microalga n.1 / microalga n.2. RP > 1, the microalga n.1 is less sensitive to the treatment than the microalga n.2. RP < 1, microalgae n.1 is more sensitive to the treatment than microalgae n.2. *N. oculata* was not included.

Pairwise comparison	Relative potencies (RP)	P-value
<i>C. muelleri</i> / <i>P. atomus</i>	1.26 ± 0.10	1.0 × 10 ⁻²
<i>C. muelleri</i> / <i>Te. chui</i>	0.32 ± 0.04	2.1 × 10 ⁻³¹
<i>C. muelleri</i> / <i>T. lutea</i>	0.11 ± 0.01	8.3 × 10 ⁻⁹¹
<i>P. atomus</i> / <i>Te. chui</i>	0.25 ± 0.028	6.0 × 10 ⁻⁴⁰
<i>P. atomus</i> / <i>T. lutea</i>	0.09 ± 0.01	8.2 × 10 ⁻⁹⁷
<i>Te. chui</i> / <i>T. lutea</i>	0.36 ± 0.04	7.0 × 10 ⁻²⁹

Our results showing a higher sensitivity of green microalgae to PACT are consistent with results by Drabkova et al. (2007), who compared the toxicity of phthalocyanines, methylene blue, tetraphenylporphyrine (S4) and hydrogen peroxide between the chlorophytes (*Pseudokirchneriella subcapitata*, *Scenedesmus quadricauda* and *Chlorella kessleri*) and cyanobacteria (*Synechococcus nidulans*, *Microcystis incerta* and *Anabaena sp.*). Although they did not report data for dark toxicity controls, they demonstrated that the green microalgae, especially *S. quadricauda*, were more sensitive to singlet oxygen than cyanobacteria.

Dark controls used in the presented study confirmed that the cationic porphyrin TMPyP was not cytotoxic to the selected microalgae. This finding is consistent with reports on bacterial cells (Malara et al., 2017b) and with previous studies on phytoplankton cells (McCullagh and Robertson, 2006b, c). In contrast Pohl et al. (2015) demonstrated that cationic corrols were cytotoxic, as negative effects on population size was evident in dark controls. They also showed that corrols induced not only reduction in phytoplankton population size when exposed to light but they also observed intracellular photooxidation of chromophore (bleaching), concluding that PS might have an effect on photosynthetic organelles.

Table 4.7: Cell surface organization and biochemical composition of the genera *Tetraselmis*, *Isochrysis*, *Chaetoceros*, *Picochlorum* and *Nannochloropsis*.

Cell Surface Organisation	Phylum/ Class	Species	Cell wall biochemical composition	References
Theca (non-mineralized)	Chlorophyta/ Prasinophyceae	<i>Tetraselmis tetrahele</i> ;	mol% of theca carbohydrates: Kdo: 54-60; MeKdo: 4; Dha: 6-8; GalA: 18-21; Ara: 1; Gal: 7; Gul: 3-4	(Becker et al., 1991; Becker et al., 1994)
Flagellar scales		<i>T. striata</i>		
Cell wall	Chlorophyta/ Trebouxiophyceae	<i>Chlorella sp.</i> ; <i>Trebouxia sp.</i>	Cellulose; Algaenans (aliphatic polymethylene polymer chains) conjugated with amides and N-alkyl-substituted pyrroles (<i>Chlorella sp.</i>) or β -galactofurans (<i>Trebouxia</i>)	(Brown and Elfman, 1983; Domozych et al., 2012)
		<i>Picochlorum atomus</i>	Cell wall 70 nm thin, homogenous appearance; Plasma membrane-associated side more electron dense; Contains structural proteins with disulphide bonds	(Porra, 2011)
Organic scales	Haptophyta/ Prymnesiophyceae	<i>Isochrysis sp.</i> ; <i>I. galbana</i> ;	Oligo-mannose-type N-glycans and/ or hybrid-type N-glycans Scales contain cellulosic microfibrils joined by acidic polysaccharides	(Espinosa et al., 2010)
Silicified frustule	Ochrophyta/ Bacillariophyceae	<i>Chaetoceros fusiformis</i> ; <i>C. affinis</i> ; <i>C. curvictetus</i> ; <i>C. decipiens</i> ; <i>C. debilis</i> ; <i>C. sociales</i>	Cell wall silica associated with proteins (frustulins, pleurifins, silaffins), polyamines and polysaccharides; Frustule polysaccharides [mol%]: Alkali-soluble: Fuc: 4-18; Gal: 7-31; Glc: 1-6; Man: 6-32; Rha: 16-52; Rib: 0-23; Xyl: 4-15 EPS polysaccharides [mol%]: Heteropolysaccharides, which can be sulphated; Fuc: 30-39; Rha: 3-35; Gal: 0-17; Man: 0-10; Xyl: 0-9; Glc: 0-5	(Gügi et al., 2015)
Cell wall	Ochrophyta/ Eustigmatophyceae	<i>Nannochloropsis gaditana</i> ; <i>N. oculata</i> ; <i>N. salina</i>	Rigid bilayered cell wall; Amino acid content: ~6% Inner part: cellulose (75% of mass balance); Dominant sugar is glucose with trace amounts of terminal sugars (Rha, Fuc, Man, NAcGlu, Gal) for crosslinking of cellulose fibrils. Outer part: 20 nm thick trilaminar sheath of algaenans, which are aliphatic C ₃₀ -straight-chain saturated hydrocarbons joined by ether bonds at terminal or one or two mid chain positions.	(Geldin et al., 1999; Scholz et al., 2014)

Abbreviations: Dha: 3-deoxy-lyxo-2-heptulosaric acid, EPS: exopolysaccharides, Fuc: fucose, Gal: galactose; GalA: galacturonic acid, Glc: glucose, Gul: gulose, Kdo: 3-deoxy-manno-2-octulosonic acid, Man: mannose, MeKdo: 3-deoxy-5-O-methyl-manno-2-octulosonic acid, NAcGlu: N-acetylglucosamine; Rha: rhamnose, Xyl: Xylos

4.3.2 Simulated algae culture contamination

Axenic microalgae cultures are commonly used in commercial application for high-value products (Olaizola, 2003; Wilkie et al., 2011) but are a less adopted practice in aquaculture, as the absence of bacteria might reduce culture growth (Cho et al., 2015; Watanabe et al., 2005) and thus, microalgal cultures can contain pathogenic bacteria. To avoid the introduction of potential pathogenic bacteria, especially in hatchery sectors where larvae are more sensitive to infections, it would be advantageous, if bacteria-free microalgae culture could be produced immediately before feeding (Vadstein, 1997).

Different methods are used to obtain an axenic microalgae culture including subculturing, serial dilution, ultrasonication, micropipetting, chemicals, ultraviolet radiation, phototaxis, osmotic pressure, electrolysis, antibiotics and most recently a combination of ultrasonication, fluorescence-activated cell sorting and micropicking (Bowyer and Skerman, 1968; Brown, 1982; Carmichael and Gorham, 1974; Connell and Cattolico, 1996; Divan and Schnoes, 1982; Gasulla et al., 2010; Hoshaw and Rosowski, 1973; Jorquera et al., 2002; Suga et al., 2011; Sykora et al., 1980; Watanabe et al., 2005; Wiedeman et al., 1964; Yim and Lee, 2004). While antibiotics is the most commonly used method, it is difficult, not economic, time-consuming (Yim and Lee, 2004) and can be toxic to microalgae cell (Sensen et al., 1993; Seoane et al., 2014; Yim and Lee, 2004; Youn and Hur, 2007). In addition, antibiotics in aquaculture should be avoided due to the potential accumulation in the environment, resistance of microorganisms and human health concerns (Alves et al., 2011a; Arijo et al., 2005; Bermúdez-Almada and Espinosa-Plascencia, 2012; Peggy and Francis-Floyd, 1996; Romalde, 2002).

The simulated microalgae culture contamination experiment presented here used different concentrations of the model bacterium *V. campbellii* ISO7 and the TMPyP-PACT-tolerant microalga *N. oculata*. Six hours of irradiation in the presence of 20 μ M TMPyP completely inactivated the luminescent signal generated by the *V. campbellii* ISO7 and killed the potential pathogen even in presence of high bacterial

loads (Figure 4.7). Plates containing CFUs showed a high diversity of colonies therefore photographic documentation of luminescence was necessary.

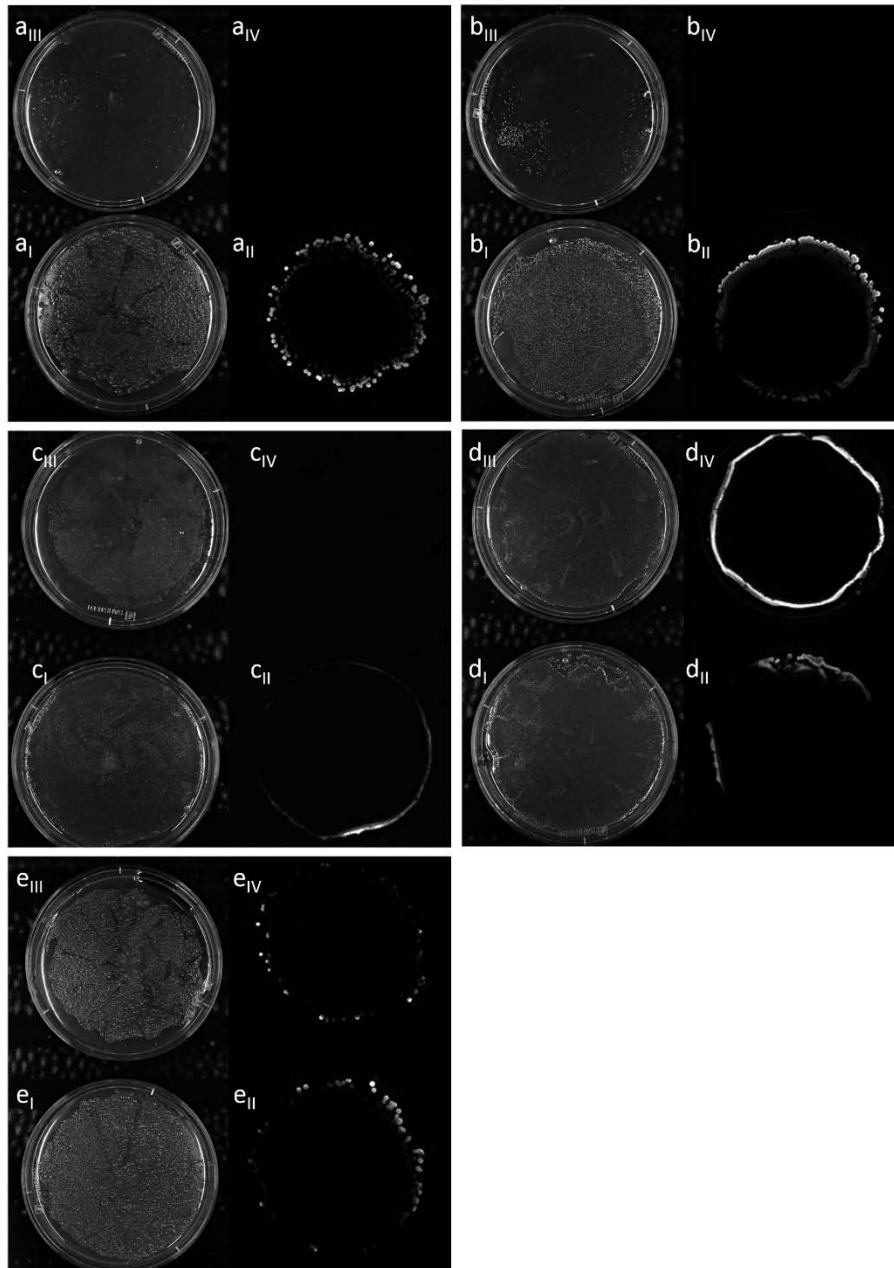


Figure 4.7: LM agar plate (I and III) and luminescent signal (II and IV) of microalgae incubated with *V. campbellii* ISO7 and 20 μM of TMPyP at the start of the experiment (I and II) and at the end (III and IV). *Nannochloropsis oculata* was mixed with *V. campbellii* ISO7 at different bacterial loads a, e) $10^3 \text{ CFU}\cdot\text{mL}^{-1}$; b) $10^5 \text{ CFU}\cdot\text{mL}^{-1}$, and c, d) $10^7 \text{ CFU}\cdot\text{mL}^{-1}$ of *V. campbellii* ISO7. Samples a-d) contained 20 μM of TMPyP and samples a-c) were irradiated, while sample d) served as the dark control. Sample e) contained no TMPyP and served as the light control.

In the presence of *N. oculata*, the luminescent signal of the model bacterium appeared to be confined to the edge of the agar plate (Figure 4.7). This unusual behaviour might be explained by respiration by *N. oculata* in the dark, potentially

leading to partial oxygen depletion, which could suppress the luminescence signal. Hence, MPN-multiplex PCR in combination with APW (*Vibrio*-selective medium) cultivation were used for detection of *Vibrio* bacteria present in the mixed culture with *N. oculata*. Regrowth did not occur in APW (data not shown) and multiplex PCR confirmed the complete inactivation of the bacterium in samples irradiated in presence of TMPyP (Figure 4.8). As expected, samples at the start of the experiment (Figure 4.8 a and b) and control samples (start and end of experiment; Figure 4.8 b, c and d) showed the presence of the model bacterium. In contrast, samples irradiated for 6 h in presence of TMPyP showed not regrowth (data not shown) and no DNA amplification (Figure 4.8 d).

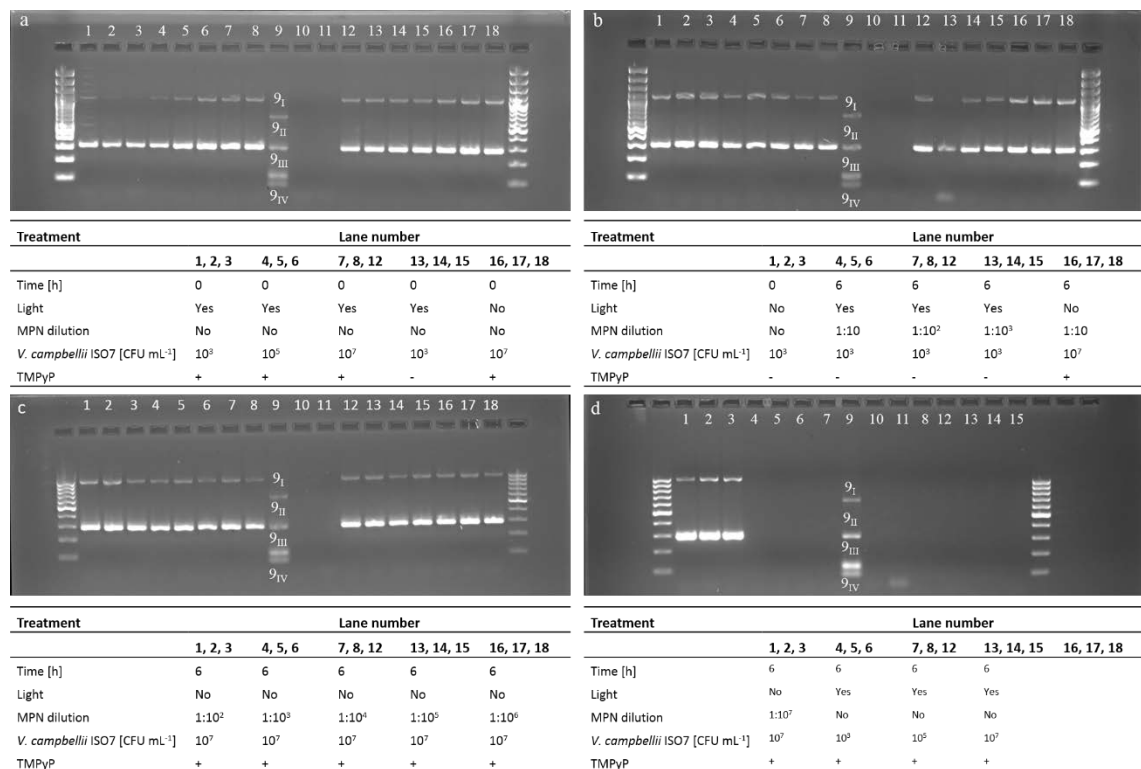


Figure 4.8: Amplification of *Vibrio* bacteria using multiplex PCR after irradiation treatments in the presence of 20 μ M TMPyP and MPN from *N. oculata* cultures mixed with *V. campbellii* ISO7 at different cell densities. For each gel, 9 = positive controls: 9_I = *Vibrio rotiferianus*, 9_{II} = *Vibrio campbellii*, 9_{III} = *Vibrio harveyi*, 9_{IV} = *Vibrio owensii*; 10 = negative control: *Vibrio fortis*; 11 = MilliQ water; All other treatments are described in below each gel.

The utilization of photosensitisers producing singlet oxygen represent, based on my knowledge, a unique study to investigate sterilization of phytoplankton for aquaculture purposes. The method used here appear to be more suitable for aquaculture purpose than the proposed method by Cho et al. (2013). In their study,

axenic culture of *C. vulgaris*, *C. sorokiniana* and *Scenedesmus* sp. were obtained after ultrasonication, fluorescence activated cell sorting (FACS), and micropicking. However, micropicking, required to grow the isolated cells in agar plate for 7 day. This is a very long period of time for the aquaculture industry considering that microalgae are used daily to feed animals.

4.4 Summary

Marine microalgae (and other microorganisms) are at the base of marine food webs and highly nutritional microalgal species are extensively used in aquaculture as live feedstock. However, difficulties in maintaining microalgae in axenic conditions make them a potential pathogen carrier and disease vectors in aquaculture ponds, which can lead to hypoxia, animal death and human intoxication. The production of $^1\text{O}_2$ and ROS in the media is a promising technique to reduce pathogens in microalgal cultures, as it creates a hostile environment for pathogenic bacteria. While photosynthetic microalgae possess ROS detoxification mechanisms, their tolerance threshold to soluble porphyrins and extracellularly generated $^1\text{O}_2$ has rarely been assessed. Therefore, the aims of this study were to test the sensitivity of aquaculture-related microalgae towards the cationic photosensitiser (TMPyP) to elucidate possible sterilization treatment of the prawn-virulent bacterium *Vibrio campbellii* ISO7. In dose- and time course response experiments, microalgae cultures were incubated with final concentrations of the soluble cationic porphyrin TMPyP of 50, 20, 10, 5, 1 and 0 μM and irradiated for up to 6 h. Light (0 μM TMPyP) and dark controls (0 and 50 μM TMPyP) were included in both dose- and time course experiments, showing that neither the irradiation conditions nor presence of TMPyP were detrimental to culture survival. Results of irradiated treatments showed that *Nannochloropsis oculata* was resilient to $^1\text{O}_2$ -produced by TMPyP even at highest dosage, whereas the entire population of *Tisochrysis lutea* (*T. lutea*), *Tetraselmis chui* (*Te. chui*), *Chaetoceros muelleri* and *Picochlorum atomus* were killed at treatment times of 6 h. Singlet oxygen sensitivity was highest in the green microalgae (*Te. chui*, *P. atomus*), followed by the diatom (*C. muelleri*), then the haptophyte (*T. lutea*) and no sensitivity in the eustigmatophyte (*N. oculata*). This indicates that microalgae cell wall biochemical composition and organisation might be responsible for the observed species-specific

$^1\text{O}_2$ sensitivity. Cell wall characteristics indicate that the theca of *Te. chui* could bind large amounts of the cationic porphyrin TMPyP due to the highly negative surface charge density generated by dominance of 2-keto sugars. Therefore, large quantities of $^1\text{O}_2$ would be generated within the theca, rendering this organism most vulnerable to $^1\text{O}_2$ treatment. In contrast, a thick covering with aliphatic C_{30} hydrocarbons crosslinked by ether bonds offers almost no binding sites for TMPyP, which explains the resilience of *N. oculata* to TMPyP-generated $^1\text{O}_2$ treatment. A simulated algae culture contamination experiment used the $^1\text{O}_2$ -resilient *N. oculata* and *V. campbellii* ISO7 at 10^3 , 10^5 , and 10^7 CFU·mL⁻¹ in the presence of 20 μM TMPyP and demonstrated complete inactivation of the free-living bacterium. This confirms the potential application of TMPyP-based PACT as a novel water treatment in aquaculture, but the suitability for sterilising microalgal feed cultures is limited to TMPyP-generated $^1\text{O}_2$ -resilient microalgae, like *N. oculata*. The TMPyP-generated $^1\text{O}_2$ treatment is not suitable for other commonly used species *T. lutea*, *Te. chui*, *C. muelleri* and *P. atomus* at TMPyP concentrations and treatment times suitable for eradicating the model bacterial pathogen.

5 Chapter 5: ⁵Suitability of PACT for Sterilisation of *Artemia* Cysts

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5.1 Introduction

Intensive aquaculture production generates water with high organic loads, ideal for fast proliferation of pathogenic bacteria. Prophylactic measures adopted to date do not efficiently prevent devastating outbreaks. Indeed, aquaculture farms experience substantial financial losses caused by bacterial contamination (Almeida et al., 2009; Austin and Austin, 2012; FAO, 2012; Saravanan et al., 2013) responsible for food poisoning (Lozano-Leon et al., 2003) and mass mortalities in early life stages of invertebrates (Cano-Gomez et al., 2010; Jithendran et al., 2010; Karunasagar et al., 1994; Muroga, 2001; Payne et al., 2006; Saoud et al., 2013; Tubiash et al., 1965) and vertebrates (Austin, 2011; Romalde, 2002; Saravanan et al., 2013; Toranzo et al., 2005; Wang et al., 2016). Potential bacterial pathogens may already be present in aquaculture water or they may be introduced *via* carriers such as live feed organisms (e.g. microalgae, brine shrimp, copepods) (Avila-Villa et al., 2011; Doucette, 1995; Pintado et al., 2014; Quiroz-Guzman et al., 2013; Tolomei et al., 2004; Verdonck et al., 1997). *Artemia* are commonly used in hatcheries due to their high nutritional value and commercial availability of storable cysts (Léger et al., 1987), but they are also considered a potential pathogen carrier in aquaculture facilities (Avila-Villa et al., 2011; Lopez-Torres and Lizarraga-Partida, 2001; Quiroz-Guzman et al., 2013; Tolomei et al.,

⁵ This chapter will be submitted to “Aquaculture” Journal.

2004). Potential pathogens have been shown to colonise *Artemia* cysts (Austin and Allen, 1982; Igarashi et al., 1989) and/or to proliferate after hatching of the nauplii (Lopez-Torres and Lizarraga-Partida, 2001).

Traditional hatching protocols for *Artemia* cysts involve a time consuming de-capsulation process using various chemicals such as sodium hypochlorite, which act as a decontaminant as well as an agent to remove the chorion (Bruggeman et al., 1980; FAO, 2007; Sorgeloos et al., 2001). A more recent approach that was recently commercialised utilises a magnetic coating around the chorion to enable collection of hatched cyst shells and unhatched cysts. This approach avoids use of the hypochlorite solution and eliminates the generation of toxic co-products and their discharge into the environment (Dhont et al., 2013).

Research on disinfection of *Artemia* has predominantly focused on chemical treatments (Douillet, 2000; Giménez et al., 2006; Høj et al., 2009; Sahul Hameed and Balasubramanian, 2000; Suantika et al., 2001; Tolomei et al., 2004), ultraviolet light (Munro et al., 1999), and administration of probiotics (Interaminense et al., 2014; Makridis et al., 2001; Makridis et al., 2000) or algae (Austin et al., 1992; Kogure et al., 1979; Olsen et al., 2000; Tolomei et al., 2004). Most of these methods have limitations. For example, antibiotics can be effective against most bacteria associated with *Artemia* but encourage selection and propagation of resistant bacterial strains. An alternative prophylactic measure to reduce the *Vibrio* load of *Artemia* is freezing nauplii for up to 48 h (Interaminense et al., 2014). However, the ability of *Vibrio* to enter a viable but not cultivable (VBNC) state during unfavourable environmental conditions, such as short exposure to freezing, is well known and can be followed by quick recovery when conditions become favourable again (Jiang and Chai, 1996; Johnston and Brown, 2002). Surprisingly, Høj et al. (2009) found that while antimicrobial treatment (formalin, Virkon® S and a mixture of antibiotics) of *Artemia* reduced their load of cultivable *Vibrio* spp, this was accompanied by a relative increase of *Vibrio*-DNA in the bacterial DNA pool, suggesting that vibrios were relatively robust to the treatment. A low-level but *Vibrio* dominated bacterial community represents a clear biosecurity risk for aquaculture hatcheries. Moreover, glycerol-associated with *Artemia* cysts can be utilised by some opportunistic bacteria including many vibrios, which can lead to their

exponential proliferation (Interaminense et al., 2014). Hence, the development of more efficient methods for disinfection of *Artemia* cysts and cultures is clearly needed.

Photodynamic antimicrobial chemotherapy (PACT), is a novel and non-specific method to kill microorganisms that is reportedly harmless to aquaculture target animals (Alves et al., 2011a; Carey, 1992; Jori and Brown, 2004; Magaraggia et al., 2006). As described in Chapter 1, PACT involves photoexcitation of a photosensitizer (PS), which triggers a sequence of photo-physical reactions that produce reactive oxygen species (ROS).

Among the various known PS that generate $^1\text{O}_2$, porphyrins have been efficiently used for disinfection of water, including drinking water (Bonnett et al., 2006), waste water (Carvalho et al., 2009; Jemli et al., 2002) and aquaculture water (Alves et al., 2011a; Malara et al., 2017a). Most porphyrins themselves, are not toxic to aquatic organisms (i.e. fish, prawns, *Artemia*) at photochemical doses (μM concentrations) (Almeida et al., 2011; Asok et al., 2012; Fabris et al., 2012; Magaraggia et al., 2006; Suzuki et al., 2000) or to humans (Almeida et al., 2011; Maisch, 2009; O'Connor et al., 2009; Ortner, 2009; Smith et al., 2009). Porphyrins are also unlikely to accumulate in the environment as a result of photobleaching after irradiation exposure (Bonnett and Martínez, 2001; Kuznetsova et al., 2010; Rotomskis et al., 1997).

The overall aim of this study was to create axenic *Artemia* cysts using PACT technology. The objectives were 1) to test and evaluate the possible concentration-dependent toxicity of PS towards *Artemia franciscana* cysts (unmodified and magnetic coated) as measured by hatching success, and 2) to test whether 20 μM of TMPyP can sterilize magnetic *Artemia* cysts seeded with the potential prawn pathogen *V. campbellii* ISO7.

5.2 Materials and Methods

5.2.1 Photosensitizer, light source irradiation condition

The PS used in this work was the cationic porphyrin TMPyP as described in previous chapters (3.2.1). Before each experiment, working stock solutions were prepared as reported in 4.2.1.2. Samples in well-plates were placed 47 cm below the light source on an orbital shaker at 150 rpm and were irradiated with continuous light

(LED 150W floodlight, irradiation of $223.8 \mu\text{mol m}^{-2}\text{s}^{-1}$) for 24 h as describe previously (3.2.3).

5.2.2 Toxicity test

5.2.2.1 *Artemia franciscana* and growth condition

Dose-response experiments to asses toxicity of PS towards *A. franciscana* cysts were conducted using two types of cysts: 1) unmodified (GSL *Artemia* Cysts, premium quality, INVE, Hoogveld, Dendermonde, Belgium), and 2) magnetically coated (SEP-Art GSL Magnetic *Artemia* Cysts A Grade, INVE).

Unmodified *Artemia* cysts, which are still used in the aquarium and aquaculture industries, require a de-capsulation step using hypochlorite solutions to dissolve the chorion before hatching (Dhont et al., 2013). This process also disinfects the cysts (Bruggeman et al., 1980; Sorgeloos et al., 1977; Sorgeloos et al., 2001). After hatching, nauplii are separated from hatched and unhatched cyst shells by passing the water, from the conical hatching tank through a $100 \mu\text{m}$ mesh where nauplii are washed and collected (FAO, 2007). On the other hand, magnetically coated *Artemia* cysts, which are commonly used nowadays in aquaculture farms (i.e. prawn hatcheries), have been created to comply with new regulations that limit discharge of toxic products such as hypochlorite solution into the environment (Dhont et al., 2013). For these cysts, it is not necessary to dissolve the chorion before hatching and cysts (hatched and unhatched) are easily separated from the nauplii using a powerful magnet (for details see www.primo.net.au).

5.2.2.2 *Experimental set up*

About 550 (0.0024 g) of either unmodified or magnetically coated cysts were hydrated in 5 ml fresh water for 1 h before 0.5 mL (~55 cysts) was transferred to 50 mL sterile centrifuge tubes (Greiner Bio-One GmbH, Austria) containing $0.22 \mu\text{m}$ filtered seawater (9.5 mL) with and without porphyrins. Final concentrations of TMPyP were 50, 20, 10, 5, 1 and $0 \mu\text{M}$. Each tube was well homogenised before transfer of 2 mL to a 24 well tissue culture plate (product # 353043, Beckton Dickinson, Franklin Lakes, New Jersey, USA) (n=5) and incubated in the dark (20 min) to facilitate porphyrin binding cysts surface and exposure to continuous light irradiation for 24 h on a rotary

shaker at 150 rpm. The final *Artemia* cysts concentration per well was approximately 5500 nauplii·L⁻¹.

Controls were created as per Table 5.1, to evaluate the possible effect of the solvent DMSO, TMPyP itself, and seawater (no DMSO) after light exposure or dark incubation. All treatments and controls were subjected to the same physical conditions during the experiment, except that dark controls were completely shielded from direct light irradiation using aluminium foil.

After 24 h incubation, newly hatched nauplii were killed by freezing and wells counted using an inverted microscope (Olympus CKX41). The average hatching success was expressed as a percentage based on counts of the number of nauplii and the total number of cysts (hatched and unhatched).

Table 5.1: Experimental design for controls used in the dose-response experiment.

Name	Porphyrin (µM)	Light exposition	DMSO (0.5%)
0 µM + DMSO	0	Yes	Yes
0 µM no DMSO	0	Yes	No
0 µM + DMSO (dark control)	0	No	Yes
0 µM no DMSO (dark control)	0	No	No
50 µM (dark control)	50	No	Yes

5.2.3 PACT disinfection of *Artemia* cysts

5.2.3.1 *Bacterium strain and growth condition*

The natural bioluminescent *Vibrio campbellii* ISO7 that showed virulence against *P. monodon* after injection as reported in 2.3.1, was used as the model bacterium. Growth conditions and media preparation were accordingly to section 3.2.2 and Table 2.1, respectively.

5.2.3.2 *Experimental set up*

Artemia franciscana cysts used in this experiment were the magnetically modified strain described in 5.2.2.1. An overnight culture of *V. campbellii* ISO7 with OD₅₇₀ adjusted to ~0.3 using sterile LM as described in 5.2.3.1 was diluted in seawater to produce final bacterial concentrations of 10³, 10⁵ and 10⁷ CFU·mL⁻¹ before adding porphyrins (final concentration 20 µM) and *A. franciscana* magnetic cysts (hydrated and diluted as described previously in 5.2.2.1). The spread-plate method (n=3) on LA

was used to verify bacterial concentrations at the start of the experiment as well as to assess bacterial viability at the end of the treatment period.

5.2.4 Statistical analysis

Statistical analyses were performed using R-Studio (RStudio Team, 2015). For all experiments, normality was assessed using the Shapiro-Wilk test (Figure A5.1) and homoscedasticity using the Levene's test (*Rcdm* package, v.2.3; Figure A5.2) (Fox, 2005, 2007). For dose-response experiments, possible treatment (porphyrin dose) effects were investigated using Van der Waerden's normal scores test (package *PMCMR* version 4.1), which is an alternative to ANOVA when normality and homogeneity of variance assumptions are violated (Conover and Iman, 1979). Differences between treatments and controls (or time) were assessed using pairwise multiple comparisons post-hoc test according to Van der Waerden (Conover and Iman, 1979).

5.3 Results and discussion

5.3.1 Toxicity test

Dose-dependent effects on cyst hatching success were seen for both unmodified (Figure 5.1) and magnetically coated (Figure 5.2) *Artemia* cysts ($p < 0.00001$ and $p < 0.0001$, respectively). Light and dark controls (0 μM , no DMSO) of unmodified and magnetically coated cysts showed low hatching success (< 40%) after 1 h rehydration followed by incubation in seawater only (Figure 5.1 and Figure 5.2, respectively). This is indicative of non-optimal hatching conditions as non-decapsulated cysts generally hatch within 24 h albeit at a somewhat lower rate than decapsulated cysts (Lavens and Sorgeloos, 1987; Lavens et al., 1986; Tunsutapanich, 1979). The low hatching rates of control cysts in the current experiment could potentially be related to insufficient resuspension of the samples leading to poor water oxygenation and/or poor cyst quality.

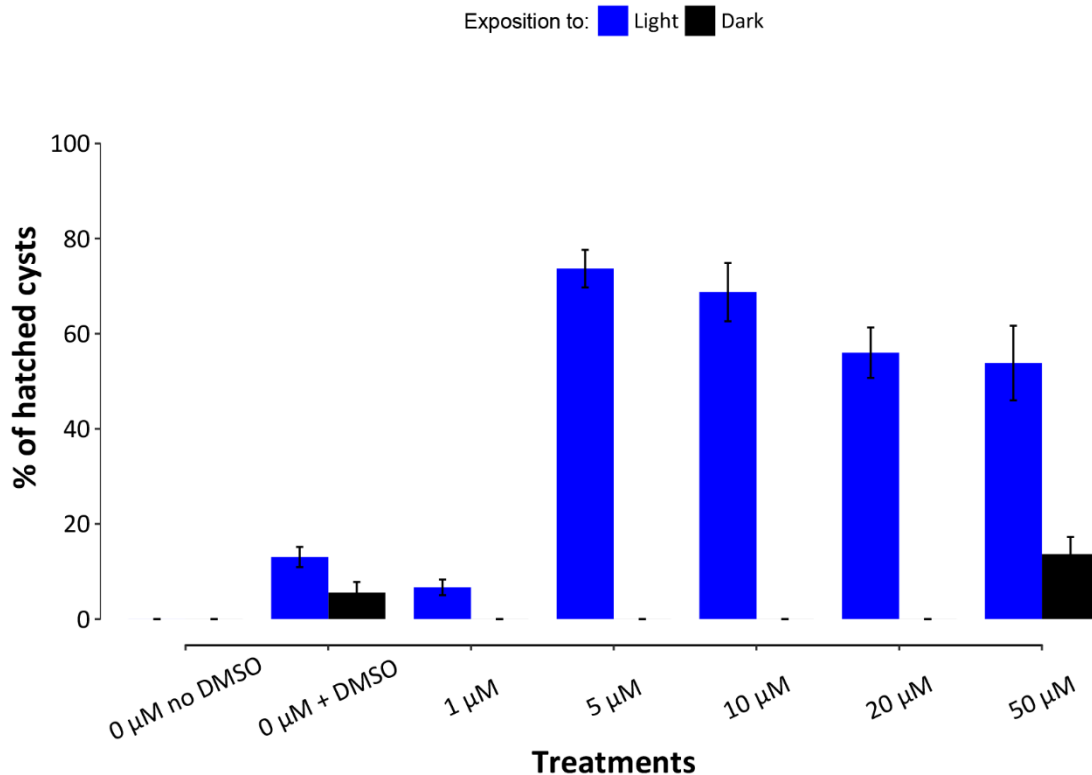


Figure 5.1: TMPyP-dose response effect on hatching rates of unmodified *Artemia franciscana* cysts in TMPyP-generated $^1\text{O}_2$ treatments and light and dark controls. The response for each treatment was calculated as percentage of the number of nauplii of each replicate divided by the total number of cysts. Error bars shows standard deviation.

For unmodified cysts exposed to 50 μM TMPYP, light irradiation improved hatching success significantly compared to the corresponding dark control ($p < 0.001$; Figure 5.1). While DMSO seemed to slightly increase hatching of unmodified *Artemia* cysts, however, the effect was not significant ($p > 0.05$; Figure 5.1). The ROS produced during PACT increased hatching success in presence of porphyrins ($> 5 \mu\text{M}$) which was significantly higher than in presence of the solvent DMSO alone ($p < 0.01$; Fig. 5.1). Hypothetically, gasses, such as $^1\text{O}_2$ and other ROS could penetrate the multi-layered chitinous structure of the cyst's shell (Robbins et al., 2010), which prevents the passage of larger molecules (Clegg et al., 1996). Therefore, it is possible that ROS produced removed or reduced the thickness of the chorion, making it easier for the nauplii to hatch in sub-optimal conditions. Similarly, an increase in hatching success was previously reported after incubating cysts with different chemicals (Bogatova and Erofeeva, 1985; Bogatova and Shmakova, 1980; Bruggeman et al., 1980; Lavens and Sorgeloos, 1987; Lavens et al., 1986; Robbins et al., 2010; Tazawa and Iwanami, 1974;

Van Stappen et al., 1998) including the ROS hydrogen peroxide (H₂O₂), which, similar to ¹O₂, leads to oxidation of organic materials (Bogatova and Shmakova, 1980).

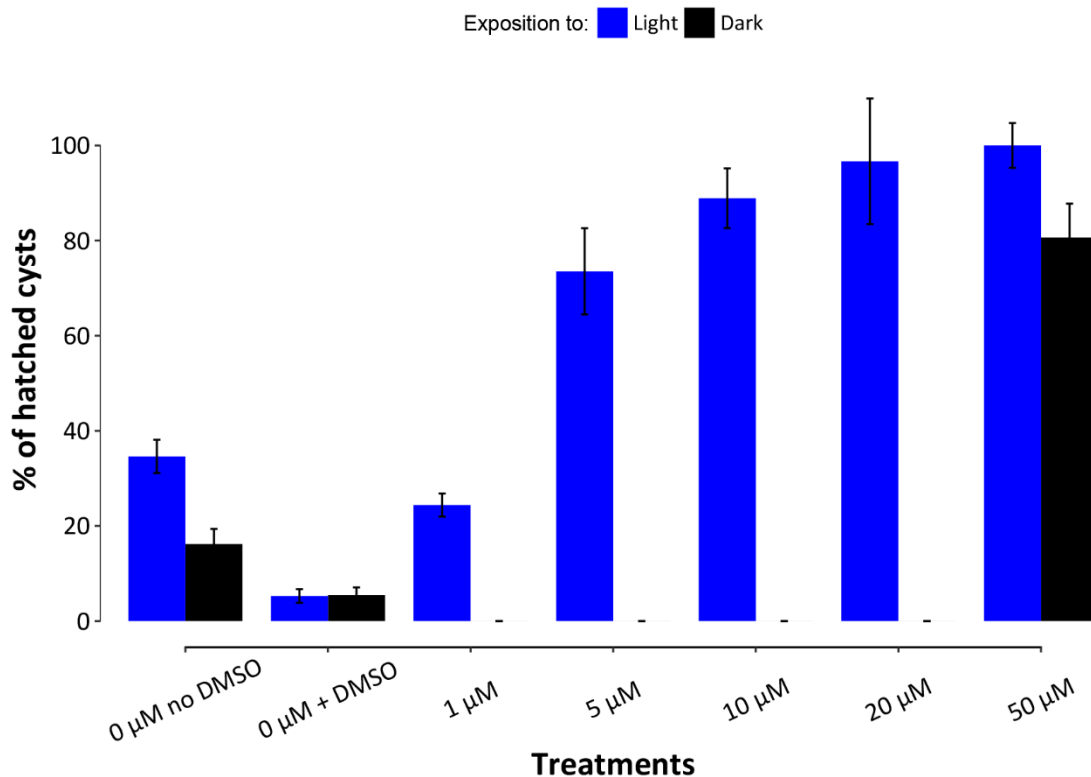


Figure 5.2: TMPyP-dose response effect on hatching rates of magnetic *Artemia franciscana* cysts in TMPyP-generated ¹O₂ treatments and light and dark controls. The response for each treatment was calculated as percentage of the sum of nauplii in each replicate divided total number of cysts. Error bars shows standard deviation.

For magnetically coated cysts, both TMPyP-generated ROS (5-50 μM, light) and TMPyP itself (50 μM dark control) significantly improved hatching success relative to light and dark controls with no TMPyP (0 μM + DMSO) ($p < 0.00001$; Figure 5.2). When irradiated, the hatching success increased in a dose-dependent manner in the presence of TMPyP (Figure 5.2). The positive effect of porphyrin without light activation was unexpected, with nearly 80% of cysts hatched in the “50 μM” dark control with no significant difference ($p = 0.27$) to the corresponding light treatment (50 μM; Figure 5.2). A possible DMSO effect can be excluded, as DMSO controls (0 μM, +DMSO) decreased rather than increased hatching rates relative to the no DMSO control (Figure 5.2). These results suggest that the porphyrin *per se* has a positive effect on hatching success of magnetic cysts, however, the TMPyP-generated ¹O₂ could potentially enhance hatching success further (~100%; Figure 5.2). TMPyP could bind to

the magnetic coating via static or magnetic attraction, causing destabilization of cyst membranes and thereby facilitating hatching. Another possible scenario is that the PACT reaction increased pH. This would be consistent with previous observations of increased hatching rates of *Artemia sinica* at pH 8.2 compared to lower pH levels (Zheng et al., 2015).

This study demonstrated that TMPyP was not toxic to *Artemia* cysts under the used experimental conditions. Previous studies of PACT treatment of *Artemia* nauplii have shown differing results. For example, no toxicity of ROS production was reported for *Artemia* nauplii exposed to rose bengal (up to 250 μM of PS, 180 min irradiation) (Asok et al., 2012); and low toxicity (below 20% mortality) was reported for nauplii exposed to the porphyrin C12 (up to 10 μM PS, 1 h irradiation) (Fabris et al., 2012) or to fluorescein (up to 250 μM of PS, up to 3 h irradiation) (Pellosi et al., 2013). Other studies have, however, reported dose- and time-dependent mortality rates of more than 20% when nauplii were exposed to 25 and 250 μM of rose bengal B, erythrosine B, eosin Y (all $^1\text{O}_2$ producers) and irradiated up to 3 h (Pellosi et al., 2013) or when exposed to 13 – 669 μM of methylene blue and irradiated for up to 1 h (Peloi et al., 2008). This suggest that sensitivity of *Artemia* nauplii to PACT is related to the nature and dose of the PS, the light type and the exposure time.

5.3.2 PACT disinfection *Artemia* cysts

After 6 h of irradiation, bacteria at concentrations up to 10^5 CFU·mL⁻¹ were completely photoinactivated ($p < 0.0001$; Table 5.2) and no luminescent colonies were seen on LA plates. For the highest bacterial concentration (2×10^7 CFU·ml⁻¹), the bacterial load was reduced to less than 10 CFU/ml (99.999% reduction). Complete sterilization was however not achieved as demonstrated by bacterial colonies, which were all luminescent on some plates. This is in contrast to the results presented in 4.3.2 where, with *N. oculata* as the model live feed organism, 10^7 CFU·mL⁻¹ of *V. campbellii* ISO7 was completely eradicated after 6 h of irradiation in presence of 20 μM of TMPyP. A possible explanation could be the difference in size between cysts and microalgae and insufficient homogenization of the samples. Indeed, magnetically coated cysts are heavier than microalgae cells and therefore require more powerful movement. Cysts, also having a greater size, might have created a shade effect on

some bacteria adjacent to the magnetic cysts and the number of PS molecules available to bind microbial cells might have been reduced due to the larger contact surface. The dark controls (+ 20 μM TMPyP) run in parallel with TMPyP light treatments did not show a reduction in number of bacteria after 24 h irradiation in the current experiment with *Artemia* (data not shown) or in the experiment with *N. oculata* (3.3).

This study has demonstrated that PACT has potential as a prophylactic method to sterilize *Artemia* cysts to use directly as a food source or to produce hatched nauplii. Decapsulated unhatched cysts are used to feed larvae of farmed animals (Celada et al., 2013; Kouba et al., 2011; Kuban et al., 1983; Ribeiro and Jones, 1998; Stael et al., 1995; Tye et al., 2014; Vandekerckhove et al., 2009), while nauplii are mainly used to feed adult animals (as live feed) or larvae (newly hatched and frozen killed nauplii) (FAO, 2007; Robertson, 2006). Killing of newly hatched *Artemia* nauplii before feeding to larvae is required mainly to limit introduction of pathogens and to facilitate predation as some larvae are not able to swim as fast as live *Artemia* nauplii (FAO, 2007; Robertson, 2006). Decapsulated unhatched cysts used as food are generally preferred to nauplii as they are less labour intensive and expensive (Bruggeman et al., 1980; Van Stappen, 1996) and have higher nutritional values (Vanhaecke et al., 1983).

Table 5.2: Bacterial CFU counts ($\text{CFU}\cdot\text{mL}^{-1}$; \pm Standard Deviation) in mixed culture with *Artemia* cysts and 20 μM TMPyP irradiated for 6 h. For each treatment CFU were counted at the start of the experiment (Time 0 h) and at the end (Time 6 h). P-values were obtained by comparing bacterial loads at the start and end of experiment, significant codes: 0 ‘***’.

Time (h)	Treatments with 20 μM TMPyP ($\text{CFU}\cdot\text{mL}^{-1}$)		
	A	B	C
0	$5.3 \pm 1.0 \times 10^3$	$2.7 \pm 1.8 \times 10^5$	$2.0 \pm 0.5 \times 10^7$
6	0 ± 0	0 ± 0	4.3 ± 5.4
p-value	***	***	***

To the best of my knowledge, no previous studies have investigated PACT sterilisation of magnetic *Artemia* cyst seeded with *Vibrio* bacteria. However, Asok et al. (2012) demonstrated that incubation of *Artemia* nauplii with 30 μM of rose bengal in presence of *V. harveyi* ($10^5 \text{CFU}\cdot\text{mL}^{-1}$) and subsequent irradiation of samples with a 150W halogen lamp caused a decrease in *Vibrio* population of about 91% after 30 min

of light exposition. Unfortunately, we cannot directly compare our result with Asok et al. (2012) because the authors did not include further details of the irradiance.

Sterilization of *Artemia* cysts using PACT would be less laborious than traditional methods used for unmodified cysts as it requires only the addition of porphyrins in the *Artemia* growing media (seawater), which will naturally degrade due to photobleaching. Currently, unmodified *Artemia* cysts require decapsulation steps with sodium hydroxide (NaOH) or calcium oxide (CaO) and calcium hypochlorite [Ca(ClO)₂], and addition of sodium thiosulphate (Na₂S₂O₃) to neutralize the chlorine and terminate the reaction (Bruggeman et al., 1980; FAO, 2007; Sorgeloos et al., 2001). This process does not sterilize but only disinfects the cysts (Bruggeman et al., 1980; Sorgeloos et al., 1977; Sorgeloos et al., 2001). Additionally, toxic by-products of the decapsulation protocol can accumulate in the water and subsequently in the environment (Dhont et al., 2013). In response, magnetic cysts were designed to comply with new regulations that limit discharge of such environmental contaminants (i.e. hypochlorite solution) (Dhont et al., 2013). While it is not necessary to dissolve the chorion of magnetic cysts before hatching and cysts (hatched and unhatched) are easily separated using a powerful magnet, this process does not completely sterilize the cysts (visit the following website for detailed information: www.primo.net.au). The photobleaching property of porphyrin used in this work prevents accumulation in the environment (Rotomskis et al., 1997). Another advantage of using porphyrins over traditional disinfection methods is that porphyrins are considered not toxic to animals at photochemical concentrations (µM) (Almeida et al., 2011; Magaraggia et al., 2006) and higher dosages (mM range) are required to cause damage in eukaryotic cells (Alves et al., 2009; Costa et al., 2008; Jemli et al., 2002; Maisch et al., 2004; Oliveira et al., 2009).

5.4 Summary

Diseases in aquaculture cause heavy financial losses and can kill the entire population of farmed animals. Live feed organisms have been recognized as one of the most important carriers of pathogens into hatchery tanks. The risk of introducing pathogenic and opportunistic bacteria such as *Vibrio* sp., can be minimized by using new antimicrobial techniques such as PACT. The advantage of using PACT is in the

economy and efficiency of the method and the photobleaching properties of the used porphyrin dyes. In the present study, meso-substitute porphyrin (TMPyP) was used as the photosensitizer and *Artemia franciscana* cysts as the model live feed organism. Possible toxic effects of TMPyP against unmodified and magnetically coated cysts was assessed by cyst hatching success (percentage) after 24 h irradiation using different porphyrin concentrations. TMPyP-generated $^1\text{O}_2$ treatment showed a dose-dependent increase in hatching success of unmodified *Artemia* cysts, while hatching success of magnetic cysts was additionally increased in the presence of TMPyP in dark controls. PACT can be also used as a disinfectant treatment for *Artemia* cysts, in fact, higher hatching success was obtained in suboptimal conditions. The disinfection capacity of $^1\text{O}_2$ -generated by TMPyP was time and bacterial load-dependent. Singlet oxygen generated by 20 μM of TMPyP completely sterilized magnetic *Artemia* cysts in the presence of 10^3 and 10^5 $\text{CFU}\cdot\text{mL}^{-1}$ of the model bacterium *Vibrio campbellii* ISO 7 after 6 h of irradiation. Even at a higher bacterial concentration of 10^7 $\text{CFU}\cdot\text{mL}^{-1}$, a more than 10^6 $\text{CFU}\cdot\text{mL}^{-1}$ reduction of bacterial load was achieved, confirming the high potential of PACT as a novel water treatment method in prawn hatcheries.

6 Chapter 6: General Discussion

6.1 Major findings and methodological considerations

This project investigated the potential of using PACT to control *Vibrio* bacteria in aquaculture. Specifically, I investigated the performance of PACT using porphyrins as a method to disinfect prawn hatchery water and common aquaculture live feed organisms such as microalgae and *Artemia*. In general, this research ascertained that PACT can be used as a substitute for, or complementary to, traditional prophylactic or preventative methods to reduce bacterial loads in aquaculture.

One of the criteria for selecting one PS in PACT studies over other compounds, is that the chosen PS should cause very limited (or no) toxicity in the dark (Detty et al., 2004; Mroz et al., 2007; Sharma et al., 2011). The PS chosen in this work (TMPyP) suggested to be a good candidate for the aquaculture industry as it was demonstrated to be not cytotoxic itself against different classes of organisms such as bacteria (chapter 3), microalgae (chapter 4) and *Artemia* cysts (chapter 5). Moreover, it was demonstrated (chapter 3) that the cationic TMPyP, as well as the tested anionic porphyrin TPPS₄, decreased in concentrations over 24 h, which was due to photobleaching. Photobleaching (photodegradation) due to the ROS produced after light exposure has been demonstrated for many porphyrins, and this prevents accumulation of these PS in the environment (Bonnett and Martínez, 2001). This also suggests that accumulation in animal tissues and associated acute cytotoxicity are unlikely to occur. However, cytotoxicity could be caused also by long term exposure (chronic) to toxic substances (i.e. porphyrins themselves or porphyrin-generated ¹O₂) and such conditions could arise in continuous treatment applications. Consistent with previous reports (Alves et al., 2009; Benov, 2015; Ergaieg and Seux, 2009; Gsponer et al., 2015; Jori and Coppellotti, 2007; Sperandio et al., 2013; Vatansever et al., 2013; Wikene et al., 2015), the tested anionic porphyrin did not impact on the survival of the model bacterium (Chapter 3), causing only a slight decline in the luminescence signal. Hence, only the cationic porphyrin was used for subsequent detailed studies.

Photodynamic antimicrobial therapy is considered a non-selective method to kill organisms due to the production of ¹O₂ as the main ROS when porphyrins are used as photosensitisers (Alves et al., 2011a; Carey, 1992; Jori and Brown, 2004; Magaraggia

et al., 2006). Like other ROS, $^1\text{O}_2$ oxidises cellular components (proteins, lipids, nucleic acids, carbohydrates) in all organisms (Skovsen et al., 2005), but effectiveness is influenced by treatment time, light intensity and spectrum, concentration, type of PS used and the ability of target organisms to withstand the toxic effect of $^1\text{O}_2$. Based on this, results presented here are subject to the conditions and concentration of the porphyrins used. Therefore, the susceptibility of a model organism such as *V. campbellii* ISO7 to PACT could differ if one or more parameters (i.e., PS concentration, light source, etc.) are modified.

Selecting a relevant model microorganism is crucial for evaluating the suitability of PACT technology for treating prawn hatchery water and feed organisms. Bioluminescence production and potential pathogenicity to target farmed animals were the selection criteria for choosing the best candidate bacterium from the Harveyi group (chapter 2). To demonstrate the virulence of bacteria towards a host animal and to verify Koch's postulates, infection experiments are often performed using oral administration, immersion or intramuscular-injection of high bacterial density suspensions (Saulnier et al., 2000) followed by re-isolation of bacteria from diseased individuals. In the current work, Koch's postulates were verified after infection experiments using intramuscular injection in prawns (Chapter 2) and identification of bacterial isolates via 2-gene MLSA, as recommended for identification of *Vibrio harveyi*-related species (Sawabe et al., 2007; Thompson et al., 2005). The selection of *Vibrio campbellii* ISO7 as the model bacterium was based on its virulence towards *P. monodon* after intra-muscular injection and its bioluminescence on LA agar.

To accurately evaluate the efficacy of the PACT treatment and ensure any cytotoxic effects of the porphyrins were detected different viability tests for bacteria and microalgae were investigated in this research. It was demonstrated that each of the investigated viability tests had advantages and limitations.

Luminescence and absorbance measurements are fast and relatively inexpensive (Alves et al., 2011a; Demidova et al., 2005). However, the methods are limited to short treatment times (natural decay of the luminescent signal; chapter 3) or require higher than naturally encountered numbers of bacteria to detect a significant signal at a specific wavelength (absorbance; chapter 3). CFU counts are reliable and

commonly used as viability tests but do not detect bacteria that are viable but not cultivatable (VBNC), potentially leading to underestimation of live bacteria (chapter 3). Furthermore, the reliability of CFU counts is limited to a range of 30 and 300 CFU per plate, hence a high number of dilutions and plates are required to ensure that accurate results are produced. In contrast, 7-day regrowth experiments are more sensitive and can detect the presence of 1 cell in the VBNC state per tube, but they are inherently time-consuming.

For many microalgae, flow cytometry based on the chlorophyll *a* auto-fluorescence signal is a suitable technique to identify microalgal populations (Sensen et al., 1993). Flow cytometry analysis requires customized settings of the parameters based on dimension of cells using forward scatter and side scatter to reduce background noise and identify the cells. This process cannot be used as a species-specific taxonomic method (Veldhuis and Kraay, 2000) but can be used to isolate single phytoplankton cells from environmental noise and bacteria present in the samples (chapter 4). In addition, it was shown that filtering the growth media reduced noise and avoided enumeration of false positives. Furthermore, the chlorophyll auto-fluorescence signal from microalgae overlaps with the PI signal when using the “red channel” (Olson and Chisholm, 1986; Van Bleijswijk and Veldhuis, 1995). It was demonstrated here, that use of the yellow fluorescence emission (583/26 nm) produced after excitation with the blue laser (488 nm) (chapter 4) allowed to accurately quantify the PI signals.

Irradiation time, intensity and spectrum, as well as the nature and concentration of the PS affect the efficiency of PACT to inactivate microorganisms (Alves et al., 2015b; Coppellotti et al., 2012; Jori et al., 2011; Vatansever et al., 2013). The research presented here showed that the killing effect of TMPyP-generated $^1\text{O}_2$ against bacteria and microalgae (chapters 3 and 4, respectively), as expected, were both dose- and time-dependent. Metabolic activity expressed as natural luminescence, as well as regrowth experiments, demonstrated that 100% lethality was achieved in the model Gram-negative bacterium *V. campbellii* ISO7 at treatment times of ~5 h or ~24 h for 20 and 1 μM TMPyP, respectively (chapter 3), while the DNA stain PI in combination with flow cytometer analysis showed that 6-h of treatment were

necessary to completely inactivate four species of microalgae (*P. atomus*, *C. muelleri*, *Te. chui* and *T. lutea*, chapter 4) as discussed in detail below.

Microalgal – and *Artemia* feed are often vectors for pathogenic bacteria in aquaculture (Lopez-Torres and Lizarraga-Partida, 2001; Murchelano and Brown, 1969; Nicolas et al., 2004; Salvesen et al., 2000), with hatcheries being particularly vulnerable due to non-specific immunity system (Bentzon-Tilia et al., 2016; Vadstein, 1997). Therefore, five microalgal species commonly used in aquaculture hatcheries were tested for their sensitivity to TMPyP itself and the TMPyP-generated $^1\text{O}_2$. Despite our understanding of microalgae ROS detoxification systems, surprisingly, four of the five microalgal species tested were highly sensitive to PACT-generated $^1\text{O}_2$, showing reduction of population size, whilst TMPyP alone was not cytotoxic (chapter 4). These results corroborate previous studies investigating toxicity of phthalocyanines (Drabkova et al., 2007; Jancula et al., 2008), tetraphenol porphyrine (Drabkova et al., 2007), methylene blue (Drabkova et al., 2007) and cationic and anionic corrols (Pohl et al., 2015) towards green algae [*Pseudokirchneriella subcapitata* (Drabkova et al., 2007; Jancula et al., 2008) *Stichococcus bacillaris* (Pohl et al., 2015), *Chlorella fusca* var. *vacuolata* (Bornhütter et al., 2016; Pohl et al., 2015), *Scenedesmus quadricauda* (Drabkova et al., 2007) and *Chlorella kessleri* (Drabkova et al., 2007)] and cyanobacteria [*Synechococcus nidulans* (Drabkova et al., 2007; Jancula et al., 2008), *Microcystis incerta* (Drabkova et al., 2007), and *Anabaena* sp. (Drabkova et al., 2007)].

In my study, the eustigmatophyte, *Nannochloropsis oculata*, was the only microalga tested that was resilient to TMPyP-generated $^1\text{O}_2$, most likely due to the nature of its cell wall with the outermost layer being composed of hydrophobic straight-chain hydrocarbons, preventing the cationic porphyrin from interacting effectively with the organisms cell wall (see Table 4.7). Recently, intra-cellular localization (using fluorescence techniques) of TMPyP in *Chlorella fusca* var. *vacuolata* cells during PACT was demonstrated (Bornhütter et al., 2016). Therefore, the species-specific microalgal susceptibility to TMPyP-generated $^1\text{O}_2$ in my work was likely to be influenced by the chemical nature of the microalgal cell walls. Similarly, this study also demonstrated that the cationic porphyrin TMPyP at concentrations of up to 50 μM was not cytotoxic to unmodified and magnetic *A. franciscana* cysts and that TMPyP-generated $^1\text{O}_2$ did

not adversely affected cyst hatching success, but surprisingly enhanced it under sub-optimal hatching conditions (chapter 5). These results are similar to findings by Asok et al. (2012) and Fabris et al. (2012) who demonstrated resistance of *Artemia* nauplii to PACT using xanthene dyes and porphyrins, respectively.

In contrast, TMPyP-generated $^1\text{O}_2$ did not cause toxicity to *Artemia* cysts confirming its non-toxicity to complex organisms at applied photochemical doses (Almeida et al., 2011; Magaraggia et al., 2006; Maisch, 2009; O'Connor et al., 2009; Ortner, 2009; Smith et al., 2009). This result is also in agreement with studies demonstrating resilience of *Artemia* nauplii to the ROS-generating dyes rose bengal and the porphyrin C12 (Asok et al., 2012; Fabris et al., 2012). Under the conditions of the research carried out in this thesis, hatching of both non-decapsulated unmodified and magnetic cysts of *A. franciscana* was facilitated by PS dosages of $> 5 \mu\text{M}$ under sub-optimal hatching conditions, but PS action differed between the two types of cysts (chapter 5). Improved hatching success was mainly due to TMPyP-generated $^1\text{O}_2$ in unmodified cysts, which is comparable to results on termination of diapause in *Artemia* when exposed to H_2O_2 (Bogatova and Erofeeva, 1985; Bogatova and Shmakova, 1980; Lavens et al., 1986; Robbins et al., 2010). In contrast, the porphyrin TMPyP *per se* improved hatching success in magnetic cysts (i.e. in dark controls) and due to TMPyP-generated $^1\text{O}_2$ (i.e. light controls). Generally, 100% hatching success is rarely achieved even under optimal conditions, as some unhatched cysts are commonly found in *Artemia* hatchery water after the hatching process (Robertson, 2006). In this context, it is noteworthy that TMPyP and TMPyP-generated $^1\text{O}_2$ achieved ~ 20 and $\sim 40\%$ under sub-optimal hatching conditions, respectively, suggesting that TMPyP-based PACT treatment can improve hatching success in sub-optimal conditions. Consequently, PACT, could be used as an alternative to the de-capsulation process currently adopted for unmodified *Artemia* cysts (Bruggeman et al., 1980; FAO, 2007; Sorgeloos et al., 2001).

The results presented here indicate that PACT may have promising applications for sterilising live feed organisms (*Artemia* cyst hatching experiment) in aquaculture, but as shown here for microalgae, the efficiency is species-specific based on the ability of live feed organisms to resist oxidative damage by $^1\text{O}_2$ produced during PACT, which

in the case of the microalgae chosen was demonstrated to be strongly influenced by the nature and architecture of their cell walls (Table 4.7). The TMPyP-based results suggest that PACT using cationic porphyrins could be used as an algicidal treatment in aquaria and aquaculture similar to published applications of the cationic porphyrin aquafrin (Schrader et al., 2010). This, however, requires more research using opportunistic and toxic species to confirm this potential. The PACT-resilient strain *N. oculata* was chosen to test possible sterilization for creating gnotobiotic algal cultures, which was previously considered as unrealistic or very difficult to obtain (Pintado et al., 2014). It was demonstrated here that a six-hour exposure to 20 μM TMPyP-generated $^1\text{O}_2$ resulted in 100% lethality of the model bacterium *V. campbellii* ISO7 and complete sterilization of the model live feed organisms *N. oculata* in mixed microalga-bacteria incubation experiments (chapter 4). In addition, the DNA of the model bacterium was not detected using molecular techniques, therefore, as DNA is not the primary target of $^1\text{O}_2$ (Almeida et al., 2015; Bonnett and Berenbaum, 2007; Dosselli et al., 2012; Maisch, 2007; Sperandio et al., 2013; Tavares et al., 2011), the absence of nucleic acids in the Multiplex PCR indicates irreversible damage to the bacterial cells.

PACT could potentially be used to sterilize *Artemia* cysts; unhatched cysts are used to feed farmed animals (Celada et al., 2013; Kouba et al., 2011; Kuban et al., 1983; Ribeiro and Jones, 1998; Stael et al., 1995; Tye et al., 2014; Vandekerckhove et al., 2009) or hatched in aquaculture facility to feed adults animals as live *Artemia* nauplii or freshly hatched and frozen killed for larval feeds (FAO, 2007; Robertson, 2006). Killing freshly hatched *Artemia* cysts is required mainly to limit contamination caused by casual introduction of pathogens and to facilitate predation as some larvae are not able to swim as fast as live *Artemia* nauplii (FAO, 2007; Robertson, 2006). The complete sterilization of *V. harveyi* by $^1\text{O}_2$ generated by rose bengal in mixed culture with *Artemia* nauplii was previously reported (Asok et al., 2012). In the current thesis (chapter 5), complete sterilization was obtained in irradiation experiments of mixed *A. franciscana* magnetic cysts and up to 10^5 $\text{CFU}\cdot\text{mL}^{-1}$ of the model pathogen *V. campbellii* ISO7 in the presence of 20 μM of TMPyP. Only very high concentrations of the bacterium (10^7 $\text{CFU}\cdot\text{mL}^{-1}$) were not sterilized, but cell numbers were substantially reduced by up to 10^6 $\text{CFU}\cdot\text{mL}^{-1}$. Cyst size itself, could have led to a self-shading effect,

sheltering bacteria attached to (or near) the cysts from irradiation in poorly suspended culture set-ups (chapter 5). This result contrasts with findings on mixed *V. campbellii* ISO7 and *N. oculata* treatments (chapter 4), where at the same bacterial concentration, PACT completely killed the model bacterium. Differences in the size of cysts and microalgae and the in homogenous suspension of cysts in the experimental set-up could explain the difference in outcomes. As cationic porphyrins are recognised to bind to Gram-negative bacterial cell walls (Demidova and Hamblin, 2005; Jori and Coppellotti, 2007; Pavani et al., 2009), higher TMPyP binding on the bigger magnetic contact surface of these cysts cannot be excluded, which would result in lower PS concentrations available for interacting with microbial cell walls.

6.2 Limitations of the research

Despite the novelty and findings discussed in section 6.1, this thesis presented some limitations primarily due to the time constraints of a PhD project and the requirement to prioritise research questions that could be addressed within the allocated time frame. Firstly, the project was limited to two potential porphyrins, one model bacterium, and five microalgae species, and as discussed below an extension of each of these groups would have enabled more general conclusions to be drawn. Secondly, the experiment targeting disinfection of *Artemia* cysts would have benefited from including regrowth assays to confirm complete sterilization. It would also have been interesting to extend the *Artemia* study to include not only cysts but also newly hatched and ongrown *Artemia* nauplii.

With regards to the range of bacterial strains, this study adds the common aquaculture pathogen *Vibrio campbellii* to the list of bacteria shown to be sensitive to PACT, which already includes *Aeromonas salmonicida* (Arrojado et al., 2011), *Enterobacter* sp. (Arrojado et al., 2011), *Enterococcus faecalis* (Arrojado et al., 2011), *Escherichia coli* (Arrojado et al., 2011), *Photobacterium damsela* subsp. *damsela* (Arrojado et al., 2011), *Photobacterium damsela* subsp. *piscicida* (Arrojado et al., 2011), *Pseudomonas* sp. (Arrojado et al., 2011), *Staphylococcus aureus* (Arrojado et al., 2011), *Vibrio anguillarum* (Arrojado et al., 2011), *V. fisheri* (Alves et al., 2011b), *V. owensii* (Malara et al., 2017b), *V. parahaemolyticus* (Arrojado et al., 2011; Malara et al., 2017b), *Flavobacterium columnare* (Schrader et al., 2010) and *Edwardsiella ictaluri*

(Schrader et al., 2010). It would be interesting to include microbial community analyses in future studies to investigate how PACT influences bacterial communities that are naturally associated with live feeds and the gastrointestinal and skin microflora of aquaculture target organisms.

The phytoplanktonic species chosen in this work represent commonly used live feed species in prawn farms (Brown and Blackburn, 2013; FAO, 2007), but do not represent the entire range of microalgae used as feed in aquaculture. Therefore, future PACT studies should extend to other commonly used species such as *Thalassiosira* sp. or *Skeletonema* sp. etc. to investigate their resilience or otherwise to TMPyP and TMPyP-generated ROS. In addition, future work should profile sterilisation effects of different classes of cationic porphyrins using the same model species and approach. A structured screening approach would allow building a PS, feed species and microbial data base, which the industry could draw from in making informed decisions on likely success of the application if adopted under their specific circumstances. For instance, there is a clear need to test the effect of different PS *per se* and their generated ROS in order to select those with lowest toxicity against microalgal feed species such as *T. lutea*, *Te. chui* and *C. muelleri*, which were shown to be sensitive to TMPyP-generated ROS.

In this study, PACT increased the hatching percentage of *Artemia* cysts that were incubated under suboptimal conditions. However, currently, there is no evidence that PACT would enhance *Artemia* hatching rates also under optimal conditions. Moreover, optimal *Artemia* cysts incubation conditions require continuous aeration (Sorgeloos, 1980; Sorgeloos et al., 1977; Van Stappen, 1996), which could lead to higher ROS production during PACT application than in the experiment described here and it would have to be investigated if higher levels of ROS could be toxic to cysts (lower hatching success) or lead to premature hatching (<18 h).

It would be interesting to extend the TMPyP *Artemia* experiments to include newly hatched nauplii and ongrown *Artemia* on their own and when seeded with a model bacterium. *Artemia* nauplii are resilient to various chemical treatments (GomezGil-Rs et al., 1994) and have been shown to adapt to extreme conditions and physiological stresses (MacRae, 2003). There is a need to find the optimal PS for

Artemia treatment, with low or no toxicity to *Artemia* nauplii, to be used in aquaculture environments. Previous studies have shown that *Artemia* are able to withstand the toxic effect of $^1\text{O}_2$ produced during PACT with different classes of PS at concentrations below 50 μM , including rose bengal (Asok et al., 2012) and porphyrins (C12; Figure 1.2b) (Fabris et al., 2012). However, higher concentrations ($>50 \mu\text{M}$) of PS such as rose bengal and other xanthene derivative dyes (Pellosi et al., 2013) and methylene blue (Peloi et al., 2008) caused nauplii mortality. This suggests that the nature and dose of PS, light type and exposure time influence the sensitivity of the target organisms.

6.3 Future directions

This thesis has demonstrated a potential for prophylactic PACT application using the cationic porphyrin TMPyP for the aquaculture industry, specifically prawn hatcheries. As a first of this kind of exploration, experiments were conducted on a small scale under controlled laboratory conditions. More research is needed to test PACT using TMPyP and other PS in a more realistic aquaculture environment (i.e. hatchery tanks and ponds), before a decision on the general usefulness of porphyrin-based PACT for the aquaculture industry can be reached.

While the thesis mainly considered use in prawn hatcheries, the technology can also be considered for use in grow-out prawn facilities (ponds). In this specific case, different factors need to be taken in account. Grow-out ponds are generally built in open environments where aeration is added in intensive and super-intensive farms (Robertson, 2006). Generally, prawns can survive at salinities between 1 and 35 $\text{g}\cdot\text{L}^{-1}$, but optimal water salinity in ponds are 15 to 25 $\text{g}\cdot\text{L}^{-1}$ with pH ranging from 7.5 to 8.5 (Robertson, 2006). PACT efficiency was shown to not be significantly different between 20 and 40 $\text{g}\cdot\text{L}^{-1}$ of salinity (Alves et al., 2011b) and pH of 5 to 8 (Alves et al., 2011b; Bertoloni et al., 1989; Watts et al., 1995), suggesting that water quality might support PACT application in ponds. However, since ponds are open environments, seasonal precipitation or evaporation could put salinity and/or pH values outside optimal ranges with unknown effects on PACT efficiency. In addition, increased water temperatures can lead to decreased levels of dissolved oxygen (Deutsch et al., 2015; Fry, 1947; Pörtner and Knust, 2007; Withers, 1992). It has been reported that PACT technology

requires a minimum of $2 \text{ mg}\cdot\text{L}^{-1}$ of dissolved oxygen to be effective (Acher et al., 1994), and there is evidence that variation in oxygen concentration can interfere with the effectiveness of PACT (Alouini and Jemli, 2001). With regards to the effect of dissolved oxygen levels on PACT efficiency, PS concentrations $< 5 \mu\text{M}$ showed no effect at about $5 \text{ mg}\cdot\text{L}^{-1}$ (Alves et al., 2011b). For aquaculture animals, optimal oxygen concentration in ponds is $4\text{-}5 \text{ mg}\cdot\text{L}^{-1}$ (Robertson, 2006) confirming again that PACT could be potentially applied to grow-out ponds but dissolved oxygen levels need to be monitored during PACT application to avoid mortality of farmed animals due to oxygen depletion. During an *in vitro* study, Jarvi et al. (2011), observed that $> 60\%$ of $^3\text{O}_2$ was consumed during PACT when meso-tetra(hydroxyphenyl)chlorin (mTHPC) PS was irradiated at a fluence rate of $55.11 \text{ mW}\cdot\text{cm}^{-2}$ for about 6 min (from 0 to $20 \text{ J}\cdot\text{cm}^{-2}$). It is, however, difficult to generalize and estimate (*in vivo*) $^3\text{O}_2$ depletion during the PACT process, as it is related to PS characteristics such as quantum yield, triplet state etc. (Jarvi et al., 2011) and also influenced by animal respiration, temperature, other organisms metabolic activity (i.e. micro-and picoplankton) and natural decay.

Water turbidity also affects the efficiency of PACT, as suspended solids reduce light penetration, and can absorb and deactivate PS (Acher et al., 1994). Therefore, under turbid conditions (seasonally changes are possible), higher concentrations of PS are required to achieve the same efficiency (Alves et al., 2011b). Photosensitizer-PACT-based $^1\text{O}_2$ generation has been reported to work at turbidity of over 25 NTU (Acher et al., 1994), which is within in the range of optimal turbidity in prawn ponds [20-30 cm Secchi disk is equivalent to 40-25 NTU; converted from (Myre and Shaw, 2006)] (Robertson, 2006). Application of PS-based PACT in open ponds is not dissimilar in design to the “sunlight disinfection plant” for wastewater effluents (Acher et al., 1994), but more studies need to be carried out in prawn outdoor ponds facilities to demonstrate similar efficiencies.

Photobleaching of PS raises questions as to whether PS break down might generate toxic products in particular under marine conditions, but there is a paucity of information in this regard. Singlet oxygen produced during PACT is associated with the photobleaching property of the PS (Bonnett and Martínez, 2001). While non- toxicity of porphyrins to animals and humans at micromolar concentrations is widely accepted

(Alves et al., 2011a; Carey, 1992; Jori and Brown, 2004; Magaraggia et al., 2006), more studies are needed to investigate possible toxic effect of by-products produced during photobleaching on larval stages of crustaceans, fish, and also feed organisms such as *Artemia*, other zooplankton and microalgae. Furthermore, as photobleaching is a time-dependent process, photosensitiser activity will also decrease with illumination time, limiting effectiveness of dosages with time. If 100% lethality on pathogenic bacteria is not achieved during the active period of the PS, re-dosing of the PS would be required, which might cause chronic toxicity in farmed animals, if treatments are performed continuously for extended periods. Therefore, PACT treatment should be carefully designed to ensure LC₁₀₀ dosages of ¹O₂ are achieved.

Bioaccumulation of toxic products or hypothetically photobleaching by-product in farmed animals cannot be completely excluded but could be limited by applying a PS attached to a solid support. Some examples of suitable solid supports include magnetic carriers, polycationic chitosan-conjugated for anionic PS, polyacrylates, porous silica, gold nanoparticles, polyacrylamide resins, silica, porous monolithic polymers etc. (Burguete et al., 2009; Manjón et al., 2010; Manjón et al., 2008; Shrestha and Kishen, 2012). However, the efficiency of this method is subject to the surface contact of the solid support with the water layers or column. Indeed, when PS are attached to a solid support, chance of binding to microbial cell walls and penetration of bacterial cells is limited (Alves et al., 2015b). PACT efficiency is not only related to binding and uptake properties of the PS (Feese et al., 2011), but also to the proximity of the generated ¹O₂ molecules to the target organisms to be eradicated (Krouit et al., 2006; Krouit et al., 2008). Therefore, to achieve similar results as reported here for suspension-based PS applications, concentrations of PS on solid supports need to be higher than for the dissolved PS (Feese et al., 2011), as diffusion of ¹O₂ is limited to relatively short distances (less than 200 nm) (Krasnovsky, 1998; Kuznetsova et al., 2011; Moan, 1990) and ¹O₂ has a short half-life in aqueous media (Ochsner, 1997; Rengifo-Herrera et al., 2005; Rodgers and Snowden, 1982). Therefore, to eradicate an acceptable level of microbial cells, close proximity of target cells with the immobilised PS is essential (Carpenter et al., 2012; Feese et al., 2011). Hence, powerful water circulation is required to avoid any “dead space” in hatchery tanks, if the immobilised PS is added to

the tanks, or alternatively, if hatchery or pond water is recirculated via a PACT column, the design of the column has to ensure sufficient contact time matched to bacterial loads. Clearly, these larger-scale experiments are required prior to applying PACT in the industry.

Moreover, PACT does not discriminate between “good” and “bad” bacterial communities and no information relating to the possible effect of PACT on the bacterial community associated with the larval gastro-intestinal system and downstream possible adverse health effects exists. This needs to be considered when PACT is applied directly to hatchery tanks (or ponds), as the bacterial community could change after PACT application. Addition of probiotics has been shown to restore beneficial bacterial communities in the digestive tract of farmed animals (Jöborn et al., 1997; Korkea-aho et al., 2012; Lazado et al., 2011; Macey and Coyne, 2006; Newaj-Fyzul et al., 2014), but PACT might require increasing the application frequency of probiotics, which also requires more research. Alternatively, PACT could be applied in a separate “PACT treatment tank” for *ad hoc* treatment, followed by transfer of the larvae (or adult animals) to normal hatchery tanks (or grow-out ponds).

When considering the application of PACT to treat live feed organisms, TMPyP-generated $^1\text{O}_2$ killed four of the five microalgae, commonly used as feed in aquaculture potentially through direct interaction of TMPyP with their cell walls, generating $^1\text{O}_2$ in immediate proximity to the cells. Further investigations should aim to confirm direct binding and/or uptake of TMPyP (Bornhütter et al., 2016) and should also investigate potential biochemical changes in the feed microalgae, as this could affect their nutritional value. As many microalgae appear to be as sensitive as the bacterial pathogen to TMPyP-generated $^1\text{O}_2$, effective sterilisation of live feed organisms using other PS should be examined. The high sensitivity of microalgae to TMPyP-generated $^1\text{O}_2$ leads to the suggestion that PACT could be more useful to reduce the risk of opportunistic or harmful algae in the aquaculture and aquaria industry but more research is needed.

As discussed above (section 6.2), effects of PACT treatment on *Artemia* nauplii appear to be PS dependent. Therefore, studies of how PACT can cause mortality in nauplii and/or changes in *Artemia* nutritional value (i.e. lipid content) requires

expansion to different PS to understand the chemical nature of the interactions of the dye with the organism to explain the different outcomes. In addition, as pointed out by Interaminense et al. (2014), glycerol released from *Artemia* cysts during hatching can support quick proliferation of opportunistic or pathogenic bacteria that can represent a potential threat for farmed animals. As the bacterial flora associated with *Artemia* cysts is diverse (Lopez-Torres and Lizarraga-Partida, 2001), it will be critical to evaluate a range of PS and their influence on the associated natural flora. Such data are required to make an informed decision by industry, as to whether or not PACT is a suitable treatment option and, if so, for which parts of the industrial process and under what conditions.

Another point that requires special attention relates to cost-benefit evaluation of potential PACT applications in the aquaculture industry. More than 20 years ago, Acher et al. (1994) estimated that a “sunlight disinfection plant” designed for wastewater treatment using methylene blue could cost about US\$ 3.95 to produce 100 m³ of disinfected water. This estimation was done considering an operation time of 8 h a day for 250 days per year and production of 200 m³·h⁻¹ of disinfected water at about 2 bar of pressure (Acher et al., 1994). However, while data generated in this research support potential benefits of PACT usage, particularly in hatcheries, the cost of the material is difficult to quantify. As mentioned previously, the efficiency of this method depends on PS dosage, the photobleaching properties of the PS, light irradiation (wavelength spectrum and intensity) and sensitivity of the target organisms. Therefore, costs of the PS relates to the quantity and efficiency of the chosen PS. For example, if irradiation were to be provided for shorter periods, a PS could stay in solution for a longer period than described for the TMPyP used here [photobleaching is irradiation time-dependent as demonstrated in 3.3.1 and by Bonnett and Martínez (2001); Hadjur et al. (1998); Kuznetsova et al. (2010); Magaraggia et al. (2006)]. Additionally, the cost of PACT in regards to PS costs could be reduced by using nano-magnet coated with porphyrins, which are retrievable from solutions, offering potential for effective recycling (Alves et al., 2014). While it is pre-mature to conduct techno-economic analyses on this new water treatment technology in aquaculture, the data generated in this PhD research show a clear bactericidal outcome and the

potential to sterilise specific microalgae and *Artemia* cysts, which has real potential to prevent bacterial outbreaks. These benefits may justify potentially higher costs (if any) associated with materials (PS, water treatment process, e.g. external tank photoreactor treatments etc.).

7 References

- Acher, A., Fischer, E., Turnheim, R., Manor, Y., 1997. Ecologically friendly wastewater disinfection techniques. *Water Research*. 31, 1398-1404.
- Acher, A.J., Fischer, E., Manor, Y., 1994. Sunlight disinfection of domestic effluents for agricultural use. *Water Research*. 28, 1153-1160.
- Aguirre-Guzman, G., Genaro Sanchez-Martinez, J., Perez-Castaneda, R., Palacios-Monzon, A., Trujillo-Rodriguez, T., Ivan de la Cruz-Hernandez, N., 2010. Pathogenicity and infection route of *Vibrio parahaemolyticus* in american white shrimp, *Litopenaeus vannamei*. *Journal of the World Aquaculture Society*. 41, 464-470.
- Aguirre-Guzmán, G., Mejia Ruíz, H., Ascencio, F., 2004. A review of extracellular virulence product of *Vibrio* species important in diseases of cultivated shrimp. *Aquaculture Research*. 35, 1395-1404.
- Ali, S.S., Shaaban, M.T., Abomohra, A., El-Safity, K., 2016. Macroalgal activity against multiple drug resistant *Aeromonas hydrophila*: A novel treatment study towards enhancement of fish growth performance. *Microbial Pathogenesis*. 101, 89-95.
- Almeida, A., Cunha, A., Gomes, N.C.M., Alves, E., Costa, L., Faustino, M.A.F., 2009. Phage therapy and photodynamic therapy: low environmental impact approaches to inactivate microorganisms in fish farming plants. *Marine Drugs*. 7, 268-313.
- Almeida, A., Cunha, A., Faustino, M.A.F., Tome, A.C., Neves, M.G.P.M.S., 2011. Chapter 5 Porphyrins as Antimicrobial Photosensitizing Agents, Photodynamic inactivation of Microbial pathogens: Medical and environmental applications. *The Royal Society of Chemistry*, pp. 83-160.
- Almeida, A., Faustino, M.A.F., Tome, J.P.C., 2015. Photodynamic inactivation of bacteria: finding the effective targets. *Future Medicinal Chemistry*. 7, 1221-1224.
- Alouini, Z., Jemli, M., 2001. Destruction of helminth eggs by photosensitized porphyrin. *Journal of Environmental Monitoring*. 3, 548-551.
- Alves, E., Carvalho, C.M.B., Tome, J.P.C., Faustino, M.A.F., Neves, M., Tome, A.C., Cavaleiro, J.A.S., Cunha, A., Mendo, S., Almeida, A., 2008. Photodynamic inactivation of recombinant bioluminescent *Escherichia coli* by cationic porphyrins under artificial and solar irradiation. *Journal of Industrial Microbiology and Biotechnology*. 35, 1447-1454.
- Alves, E., Costa, L., Carvalho, C.M., Tome, J.P., Faustino, M.A., Neves, M.G., Tome, A.C., Cavaleiro, J.A., Cunha, A., Almeida, A., 2009. Charge effect on the photoinactivation of Gram-negative and Gram-positive bacteria by cationic meso-substituted porphyrins. *BMC Microbiology*. 9, 70-82.
- Alves, E., Costa, L., Cunha, A., Faustino, M.A.F., Neves, M.G.P.M.S., Almeida, A., 2011a. Bioluminescence and its application in the monitoring of antimicrobial photodynamic therapy. *Applied Microbiology and Biotechnology*. 92, 1115-1128.

- Alves, E., Faustino, M.A., Tome, J.P., Neves, M.G., Tome, A.C., Cavaleiro, J.A., Cunha, A., Gomes, N.C., Almeida, A., 2011b. Photodynamic antimicrobial chemotherapy in aquaculture: photoinactivation studies of *Vibrio fischeri*. PLoS One. 6, e20970.
- Alves, E., Faustino, M.A.F., Tome, J.P.C., Neves, M., Tome, A.C., Cavaleiro, J.A.S., Cunha, A., Gomes, N.C.M., Almeida, A., 2013. Nucleic acid changes during photodynamic inactivation of bacteria by cationic porphyrins. Bioorganic and Medicinal Chemistry. 21, 4311-4318.
- Alves, E., Rodrigues, J.M.M., Faustino, M.A.F., Neves, M.G.P.M.S., Cavaleiro, J.A.S., Lin, Z., Cunha, A., Nadais, M.H., Tome, J.P.C., Almeida, A., 2014. A new insight on nanomagnet-porphyrin hybrids for photodynamic inactivation of microorganisms. Dyes and Pigments. 110, 80-88.
- Alves, E., Esteves, A.C., Correia, A., Cunha, A., Faustino, M.A.F., Neves, M., Almeida, A., 2015a. Protein profiles of *Escherichia coli* and *Staphylococcus warneri* are altered by photosensitization with cationic porphyrins. Photochemical and Photobiological Sciences. 14, 1169-1178.
- Alves, E., Faustino, M.A.F., Neves, M.G.P.M.S., Cunha, A., Nadais, H., Almeida, A., 2015b. Potential applications of porphyrins in photodynamic inactivation beyond the medical scope. Journal of Photochemistry and Photobiology C: Photochemistry Reviews. 22, 34-57.
- Arenas, Y., Monro, S., Shi, G., Mandel, A., McFarland, S., Lilge, L., 2013. Photodynamic inactivation of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* with Ru(II)-based type I/type II photosensitizers. Photodiagnosis and photodynamic therapy. 10, 615-625.
- Arijo, S., Rico, R., Chabrilion, M., Diaz-Rosales, P., Martinez-Manzanares, E., Balebona, M.C., Magarinos, B., Toranzo, A.E., Morinigo, M.A., 2005. Effectiveness of a divalent vaccine for sole, *Solea senegalensis* (Kaup), against *Vibrio harveyi* and *Photobacterium damsela subsp piscicida*. Journal of Fish Diseases. 28, 33-38.
- Arrojado, C., Pereira, C., Tome, J.P.C., Faustino, M.A.F., Neves, M.G.P.M.S., Tome, A.C., Cavaleiro, J.A.S., Cunha, A., Calado, R., Gomes, N.C.M., Almeida, A., 2011. Applicability of photodynamic antimicrobial chemotherapy as an alternative to inactivate fish pathogenic bacteria in aquaculture systems. Photochemical and Photobiological Sciences. 10, 1691-1700.
- Asok, A., Arshad, E., Jasmin, C., Pai, S.S., Singh, I.S.B., Mohandas, A., Anas, A., 2012. Reducing *Vibrio* load in *Artemia* nauplii using antimicrobial photodynamic therapy: a promising strategy to reduce antibiotic application in shrimp larviculture. Microbial Biotechnology. 5, 59-68.
- Austin, B., Allen, D.A., 1982. Microbiology of laboratory-hatched brine shrimp (*Artemia*). Aquaculture. 26, 369-383.
- Austin, B., Baudet, E., Stobie, M., 1992. Inhibition of bacterial fish pathogens by *Tetraselmis suecica*. Journal of Fish Diseases. 15, 55-61.
- Austin, B., Zhang, X.H., 2006. *Vibrio harveyi*: a significant pathogen of marine vertebrates and invertebrates. Letters in Applied Microbiology. 43, 119-124.

- Austin, B., 2011. Taxonomy of bacterial fish pathogens. *Veterinary Research*. 42, 13.
- Austin, B., Austin, D.A., 2012. *Bacterial fish pathogens: Disease in farmed and wild fish*, Fifth Edition. Springer Dordrecht Heidelberg New York, London, 978-94-007-4883-5.
- Avila-Villa, L.A., Martínez-Porchas, M., Gollas-Galván, T., López-Elías, J.A., Mercado, L., Murguía-López, Á., Mendoza-Cano, F., Hernández-López, J., 2011. Evaluation of different microalgae species and *Artemia* (*Artemia franciscana*) as possible vectors of necrotizing hepatopancreatitis bacteria. *Aquaculture*. 318, 273-276.
- Awad, M.M., Tovmasyan, A., Craik, J.D., Batinic-Haberle, I., Benov, L.T., 2016. Important cellular targets for antimicrobial photodynamic therapy. *Applied microbiology and biotechnology*. 100, 7679-7688.
- Babilas, P., Schreml, S., Landthaler, M., Szeimies, R.M., 2010. Photodynamic therapy in dermatology: state-of-the-art. *Photodermatology, photoimmunology and photomedicine*. 26, 118-132.
- Bacellar, I.O.L., Tsubone, T.M., Pavani, C., Baptista, M.S., 2015. Photodynamic efficiency: From molecular photochemistry to cell death. *International Journal of Molecular Sciences*. 16, 20523-20559.
- Beacham, T.A., Bradley, C., White, D.A., Bond, P., Ali, S.T., 2014. Lipid productivity and cell wall ultrastructure of six strains of *Nannochloropsis*: Implications for biofuel production and downstream processing. *Algal Research*. 6, 64-69.
- Becker, B., Becker, D., Kamerling, J.P., Melkonian, M., 1991. 2-keto-sugar acids in green flagellates: a chemical marker for prasinophycean scales. *Journal of Phycology*. 27, 498-504.
- Becker, B., Marin, B., Melkonian, M., 1994. Structure, composition, and biogenesis of prasinophyte cell coverings. *Protoplasma*. 181, 233-244.
- Bendif, E., Probert, I., Schroeder, D.C., de Vargas, C., 2013. On the description of *Tisochrysis lutea* gen. nov sp nov and *Isochrysis nuda* sp nov in the *Isochrysidales*, and the transfer of Dicrateria to the Prymnesiales (Haptophyta). *Journal of Applied Phycology*. 25, 1763-1776.
- Benov, L., 2015. Photodynamic therapy: current status and future directions. *Medical Principles and Practice*. 24, 14-28.
- Bentzon-Tilia, M., Sonnenschein, E.C., Gram, L., 2016. Monitoring and managing microbes in aquaculture - Towards a sustainable industry. *Microbial Biotechnology*. 9, 576-584.
- Berg, K., Folini, M., Prasmickaite, L., Selbo, P.K., Bonsted, A., Engesaeter, B.O., Zaffaroni, N., Weyergang, A., Dietze, A., Maeldandsmo, G.M., Wagner, E., Norum, O.J., Hogset, A., 2007. Photochemical internalization: a new tool for drug delivery. *Current pharmaceutical biotechnology*. 8, 362-372.
- Bermúdez-Almada, M.C., Espinosa-Plascencia, A., 2012. The use of antibiotics in shrimp farming, in: Carvalho, D.E. (Ed.), *Health and Environment in Aquaculture*. InTech, pp. 199-2014.

- Berney, M., Hammes, F., Bosshard, F., Weilenmann, H.-U., Egli, T., 2007. Assessment and interpretation of bacterial viability by using the LIVE/DEAD BacLight kit in combination with flow cytometry. *Applied and Environmental Microbiology*. 73, 3283-3290.
- Bertoloni, G., Reddi, E., Gatta, M., Burlini, C., Jori, G., 1989. Factors influencing the haematoporphyrin-sensitized photoinactivation of *Candida albicans*. *Journal of general microbiology*. 135, 957-966.
- Bertoloni, G., Rossi, F., Valduga, G., Jori, G., Ali, H., Lier, J., 1992. Photosensitizing activity of water- and lipid-soluble phthalocyanines on prokaryotic and eukaryotic microbial cells. *Microbios*. 71, 33 - 46.
- Bogatova, I., Shmakova, Z., 1980. Activation of diapausing *Artemia salina* eggs. *Hydrobiological Journal*. 16, 108-110.
- Bogatova, I., Erofeeva, G., 1985. Incubation of *Artemia salina* resting eggs without preliminary stimulation. *Hydrobiologicheskij Journal*. 21, 52-56.
- Bolch, C.J.S., Subramanian, T.A., Green, D.H., 2011. The toxic dinoflagellate *Gymnodinium catenatum* (Dinophyceae) requires marine bacteria for growth. *Journal of Phycology*. 47, 1009-1022.
- Bonnett, R., Martínez, G., 2001. Photobleaching of sensitizers used in photodynamic therapy. *Tetrahedron*. 57, 9513-9547.
- Bonnett, R., Krysteva, M.A., Lalov, I.G., Artarsky, S.V., 2006. Water disinfection using photosensitizers immobilized on chitosan. *Water Research*. 40, 1269-1275.
- Bonnett, R., Berenbaum, M., 2007. Porphyrins as Photosensitizers, *Ciba Foundation Symposium 146 - Photosensitizing Compounds: Their Chemistry, Biology and Clinical Use*. John Wiley & Sons, Ltd., pp. 40-59.
- Bornhütter, T., Pohl, J., Fischer, C., Saltsman, I., Mahammed, A., Gross, Z., Röder, B., 2016. Development of singlet oxygen luminescence kinetics during the photodynamic inactivation of green algae. *Molecules*. 21, 485.
- Borowitzka, M.A., 1995. Microalgae as sources of pharmaceuticals and other biologically active compounds. *Journal of Applied Phycology*. 7, 3-15.
- Borowitzka, M.A., Moheimani, N.R., 2010. Sustainable biofuels from algae. *Mitigation and Adaptation Strategies for Global Change*. 18, 13-25.
- Bowyer, J., Skerman, V., 1968. Production of axenic cultures of soil-borne and endophytic blue-green algae. *Microbiology*. 54, 299-306.
- Boyle, R., Dolphin, D., 1996. Structure and biodistribution relationships of photodynamic sensitizers. *Photochemistry and Photobiology*. 64, 469 - 485.
- Bozzini, G., Colin, P., Betrouni, N., Nevoux, P., Ouzzane, A., Puech, P., Villers, A., Mordon, S., 2012. Photodynamic therapy in urology: What can we do now and where are we heading? *Photodiagnosis and Photodynamic Therapy*. 9, 261-273.
- Brovko, L., 2010. Photodynamic treatment: A new efficient alternative for surface sanitation. *Advances in food and nutrition research*. 61, 119-147.

- Brown, L.M., 1982. Production of axenic cultures of algae by an osmotic method. *Phycologia*. 21, 408-410.
- Brown, L.M., Elfman, B., 1983. Is autospore formation a feature of *Nannochloris*? *Canadian Journal of Botany*. 61, 2647-2657.
- Brown, M.R., Blackburn, S.I., 2013. Live microalgae as feeds in aquaculture hatcheries, in: Allan, G., Burnell, G. (Eds.), *Advances in Aquaculture Hatchery Technology*. Woodhead Publ Ltd, Abington, Cambridge, Uk, pp. 117-156.
- Bruggeman, E., Sorgeloos, P., Vanhaecke, P., 1980. Improvements in the decapsulation technique of *Artemia* cysts, in: Persoon, G., Sorgeloos, P., Roels, O., Jaspers, E. (Eds.), *The brine shrimp Artemia*. Universa Press, Wetteren, Belgium, pp. 260-269.
- Buchholz, J., Walt, H., 2013. Veterinary photodynamic therapy: A review. *Photodiagnosis and Photodynamic Therapy*. 10, 342-347.
- Burguete, M.I., Galindo, F., Gavara, R., Luis, S.V., Moreno, M., Thomas, P., Russell, D.A., 2009. Singlet oxygen generation using a porous monolithic polymer supported photosensitizer: potential application to the photodynamic destruction of melanoma cells. *Photochemical and photobiological sciences*. 8, 37-44.
- Busico-Salcedo, N., Owens, L., 2013. Virulence changes to *Harveyi* clade bacteria infected with bacteriophage from *Vibrio owensii*. *Indian Journal of Virology*. 24, 180-187.
- Butcher, R.W., 1952. Contributions to our knowledge of the smaller marine algae. *Journal of the Marine Biology Association of the United Kingdom*. 31, 175-191.
- Butcher, R.W., 1959. An introductory account of the smaller algae of British coastal waters. Part I: Introduction and Chlorophyceae. *Fisheries Investigations*, London,
- Cabello, F.C., 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environmental Microbiology*. 8, 1137-1144.
- Cadet, J., Ravanat, J.L., Martinez, G.R., Medeiros, M.H.G., Di Mascio, P., 2006. Singlet oxygen oxidation of isolated and cellular DNA: Product formation and mechanistic insights. *Photochemistry and Photobiology*. 82, 1219-1225.
- Cadet, J., Douki, T., Ravanat, J.L., 2010. Oxidatively generated base damage to cellular DNA. *Free Radical Biology and Medicine*. 49, 9-21.
- Calzavara-Pinton, P., Rossi, M.T., Sala, R., Venturini, M., 2012. Photodynamic antifungal chemotherapy. *Photochemistry and photobiology*. 88, 512-522.
- Cano-Gomez, A., Bourne, D.G., Hall, M.R., Owens, L., Høj, L., 2009. Molecular identification, typing and tracking of *Vibrio harveyi* in aquaculture systems: Current methods and future prospects. *Aquaculture*. 287, 1-10.
- Cano-Gomez, A., Goulden, E.F., Owens, L., Høj, L., 2010. *Vibrio owensii* sp nov., isolated from cultured crustaceans in Australia. *FEMS Microbiology Letters*. 302, 175-181.
- Cano-Gomez, A., Høj, L., Owens, L., Andreakis, N., 2011. Multilocus sequence analysis provides basis for fast and reliable identification of *Vibrio harveyi*-related species

- and reveals previous misidentification of important marine pathogens. *Systematic and Applied Microbiology*. 34, 561-565.
- Cano-Gomez, A., 2012. Molecular identification of *Vibrio harveyi*-related bacteria and *Vibrio owensii* sp. nov., pathogenic to larvae of the ornate spiny lobster *Panulirus ornatus*. James Cook University, Australia, pp. 1-191.
- Cano-Gomez, A., Høj, L., Owens, L., Baillie, B.K., Andreakis, N., 2015. A multiplex PCR-based protocol for identification and quantification of *Vibrio harveyi*-related species. *Aquaculture*. 437, 195-200.
- Carey, J.H., 1992. An introduction to advanced oxidation processes (AOP) for destruction of organics in wastewater. *Water Pollution Research Journal of Canada*. 27, 1-21.
- Carmichael, W.W., Gorham, P.R., 1974. An improved method for obtaining axenic clones of planktonic blue-green algae^{1,2}. *Journal of Phycology*. 10, 238-240.
- Carpenter, B.L., Feese, E., Sadeghifar, H., Argyropoulos, D.S., Ghiladi, R.A., 2012. Porphyrin-cellulose nanocrystals: a photobactericidal material that exhibits broad spectrum antimicrobial activity. *Photochemistry and photobiology*. 88, 527-536.
- Carre, V., Gaud, O., Sylvain, I., Bourdon, O., Spiro, M., Blais, J., Granet, R., Krausz, P., Guilloton, M., 1999. Fungicidal properties of meso-arylglycosylporphyrins: influence of sugar substituents on photoinduced damage in the yeast *Saccharomyces cerevisiae*. *Journal of Photochemistry and Photobiology B: Biology*. 48, 57-62.
- Carvalho, C.M.B., Gomes, A., Fernandes, S.C.D., Prata, A.C.B., Almeida, M.A., Cunha, M.A., Tome, J.P.C., Faustino, M.A.F., Neves, M., Tome, A.C., Cavaleiro, J.A.S., Lin, Z., Rainho, J.P., Rocha, J., 2007. Photoinactivation of bacteria in wastewater by porphyrins: Bacterial beta-galactosidase activity and leucine-uptake as methods to monitor the process. *Journal of Photochemistry and Photobiology B: Biology*. 88, 112-118.
- Carvalho, C.M.B., Tome, J.P.C., Faustino, M.A.F., Neves, M., Tome, A.C., Cavaleiro, J.A.S., Costa, L., Alves, E., Oliveira, A., Cunha, A., Almeida, A., 2009. Antimicrobial photodynamic activity of porphyrin derivatives: potential application on medical and water disinfection. *Journal of Porphyrins and Phthalocyanines*. 13, 574-577.
- Castano, A.P., Demidova, T.N., Hamblin, M.R., 2004. Mechanisms in photodynamic therapy: part one-photosensitizers, photochemistry and cellular localization. *Photodiagnosis and Photodynamic Therapy*. 1, 279-293.
- Castano, A.P., Demidova, T.N., Hamblin, M.R., 2005. Mechanisms in photodynamic therapy: part two—cellular signaling, cell metabolism and modes of cell death. *Photodiagnosis and photodynamic therapy*. 2, 1-23.
- Celada, J.D., García, V., Carral, J.M., Sáez-Royuela, M., González, R., González, Á., 2013. Decapsulated *Artemia* cysts of different quality (high or low hatch-rate) as direct food for tench (*Tinca tinca* L.) larvae. *Aquaculture Research*. 44, 167-175.
- Chambers, J., Freeny, A., Heiberger, R., 1992. Chapter 5 of statistical models in S, in: Brooks/Cole, W. (Ed.), *Analysis of Variance; Designed Experiments*. Pacific Grove.

- Chiu, S.T., Wong, S.L., Shiu, Y.L., Chiu, C.H., Guei, W.C., Liu, C.H., 2016. Using a fermented mixture of soybean meal and earthworm meal to replace fish meal in the diet of white shrimp, *Penaeus vannamei* (Boone). *Aquaculture Research*. 47, 3489-3500.
- Cho, D.-H., Ramanan, R., Kim, B.-H., Lee, J., Kim, S., Yoo, C., Choi, G.-G., Oh, H.-M., Kim, H.-S., 2013. Novel approach for the development of axenic microalgal cultures from environmental samples. *Journal of Phycology*. 49, 802-810.
- Cho, D.-H., Ramanan, R., Heo, J., Lee, J., Kim, B.-H., Oh, H.-M., Kim, H.-S., 2015. Enhancing microalgal biomass productivity by engineering a microalgal–bacterial community. *Bioresource Technology*. 175, 578-585.
- Chrisolite, B., Thiyagarajan, S., Alavandi, S.V., Abhilash, E.C., Kalaimani, N., Vijayan, K.K., Santiago, T.C., 2008. Distribution of luminescent *Vibrio harveyi*, and their bacteriophages in a commercial shrimp hatchery in South India. *Aquaculture*. 275, 13-19.
- Clegg, J.S., Drinkwater, L.E., Sorgeloos, P., 1996. The metabolic status of diapause embryos of *Artemia franciscana* (SFB). *Physiological zoology*. 69, 49-66.
- Colwell, R.R., 1984. *Vibrios in the environment*. John Wiley & Sons, Ltd., 1099-1263.
- Connell, L., Cattolico, R.A., 1996. Fragile algae: axenic culture of field-collected samples of *Heterosigma carterae*. *Marine Biology*. 125, 421-426.
- Conover, W., Iman, R., 1979. On multiple-comparisons procedures. Los Alamos Sci. Lab. Tech. Rep. LA-7677-MS.
- Coppellotti, O., Fabris, C., Soncin, M., Magaraggia, M., Camerin, M., Jori, G., Guidolin, L., 2012. Porphyrin photosensitised processes in the prevention and treatment of water- and vector-borne diseases. *Current Medicinal Chemistry*. 19, 808-819.
- Cormick, M.P., Alvarez, M.G., Rovera, M., Durantini, E.N., 2009. Photodynamic inactivation of *Candida albicans* sensitized by tri- and tetra-cationic porphyrin derivatives. *European Journal of Medicinal Chemistry*. 44, 1592-1599.
- Costa, L., Alves, E., Carvalho, C., Tome, J., Faustino, M., Neves, M., Tome, A., Cavaleiro, J., Cunha, A., Almeida, A., 2008. Sewage bacteriophage photoinactivation by cationic porphyrins: a study of charge effect. *Photochemical and Photobiological Sciences*. 7, 415 - 422.
- Costa, L., Faustino, M.A.F., Neves, M., Cunha, A., Almeida, A., 2012. Photodynamic inactivation of mammalian viruses and bacteriophages. *Viruses*. 4, 1034-1074.
- Cotter, T.G., 2009. Apoptosis and cancer: the genesis of a research field. *Nature Reviews: Cancer*. 9, 501-507.
- Croft, M.T., Lawrence, A.D., Raux-Deery, E., Warren, M.J., Smith, A.G., 2005. Algae acquire vitamin B12 through a symbiotic relationship with bacteria. *Nature*. 438, 90-93.
- Dahl, T.A., Midden, W.R., Hartman, P.E., 1987. Pure singlet oxygen cytotoxicity for bacteria. *Photochemistry and Photobiology*. 46, 345-352.

- Daintith, M., 1996. Rotifers and artemia for marine aquaculture : a training guide. University of Tasmania, Tasmania,
- Darshaneeruwandeeepika, H.A., Sanjeeva Prasad Jayaweera, T., Paban Bhowmick, P., Karunasagar, I., Bossier, P., Defoirdt, T., 2012. Pathogenesis, virulence factors and virulence regulation of vibrios belonging to the Harveyi-clade. *Reviews in Aquaculture*. 4, 59-74.
- Darzynkiewicz, Z., Li, X., Gong, J., 1994. Assays of cell viability: Discrimination of cells dying by apoptosis, in: Darzynkiewicz, Z., Robinson, J.P., Crissman, H.A. (Eds.), *Methods in Cell Biology; Flow cytometry, Part A, Second edition*. Academic Press, Inc., San Diego, California, USA, pp. 15-38.
- Davies, M.J., 2003. Singlet oxygen-mediated damage to proteins and its consequences. *Biochemical and Biophysical Research Communications*. 305, 761-770.
- Davies, M.J., 2004. Reactive species formed on proteins exposed to singlet oxygen. *Photochemical and Photobiological Sciences*. 3, 17-25.
- De Souza, L.M., Pratavieira, S., Inada, N.M., Kurachi, C., Corbi, J., Guimaraes, F.E.G., Bagnato, V.S., 2014. Efficacy of photodynamic therapy against larvae of *Aedes aegypti*: Confocal microscopy and fluorescence-lifetime imaging, in: Farkas, D.L., Nicolau, D.V., Leif, R.C. (Eds.), *Imaging, Manipulation, and Analysis of Biomolecules, Cells, and Tissues XII*. Spie-Int Soc Optical Engineering, Bellingham.
- De Zwart, D., Slooff, W., 1983. The microtox as an alternative assay in the acute toxicity assessment of water pollutants. *Aquatic Toxicology (Amsterdam)*. 4, 129-138.
- Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., Verstraete, W., 2006. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. *Aquaculture*. 261, 804-808.
- Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2007a. Quorum sensing and quorum quenching in *Vibrio harveyi*: lessons learned from in vivo work. *ISME Journal*. 2, 19-26.
- Defoirdt, T., Halet, D., Vervaeren, H., Boon, N., Van de Wiele, T., Sorgeloos, P., Bossier, P., Verstraete, W., 2007b. The bacterial storage compound poly-beta-hydroxybutyrate protects *Artemia franciscana* from pathogenic *Vibrio campbellii*. *Environmental Microbiology*. 9, 445-452.
- Defoirdt, T., Verstraete, W., Bossier, P., 2008. Luminescence, virulence and quorum sensing signal production by pathogenic *Vibrio campbellii* and *Vibrio harveyi* isolates. *Journal of Applied Microbiology*. 104, 1480-1487.
- Defoirdt, T., Boon, N., Bossier, P., 2010. Can Bacteria Evolve Resistance to Quorum Sensing Disruption? *Plos Pathogens*. 6.
- Defoirdt, T., Sorgeloos, P., 2012. Monitoring of *Vibrio harveyi* quorum sensing activity in real time during infection of brine shrimp larvae. *ISME Journal*. 6, 2314-2319.
- Demidova, T.N., Hamblin, M.R., 2004. Photodynamic therapy targeted to pathogens. *International Journal of Immunopathology and Pharmacology*. 17, 245-254.
- Demidova, T.N., Gad, F., Zahra, T., Francis, K.P., Hamblin, M.R., 2005. Monitoring photodynamic therapy of localized infections by bioluminescence imaging of

- genetically engineered bacteria. *Journal of Photochemistry and Photobiology B: Biology*. 81, 15-25.
- Demidova, T.N., Hamblin, M.R., 2005. Effect of cell-photosensitizer binding and cell density on microbial photoinactivation. *Antimicrobial Agents and Chemotherapy*. 49, 2329-2335.
- Denis, T.G.S., Dai, T., Izikson, L., Astrakas, C., Anderson, R.R., Hamblin, M.R., Tegos, G.P., 2011. All you need is light. Antimicrobial photoinactivation as an evolving and emerging discovery strategy against infectious disease. *Virulence*. 2, 509-520.
- Department of Agriculture Fisheries and Forestry, 2013. The aquaculture industry in Australia. 12 Aug 2016. Available from: <http://www.daff.gov.au>.
- Department of Primary Industries and Fisheries, 2008. Australian Prawn Farming – An Industry Development Plan 2008-2010. in: Fisheries, D.o.P.I.a. (Ed.). The State of Queensland: Brisbane.
- DeRosa, M.C., Crutchley, R.J., 2002. Photosensitized singlet oxygen and its applications. *Coordination Chemistry Reviews*. 233, 351-371.
- Detty, M.R., Gibson, S.L., Wagner, S.J., 2004. Current clinical and preclinical photosensitizers for use in photodynamic therapy. *Journal of medicinal chemistry*. 47, 3897-3915.
- Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O., Huey, R.B., 2015. Climate change tightens a metabolic constraint on marine habitats. *Science*. 348, 1132-1135.
- Dhont, J., Dierckens, K., Støttrup, J., Van Stappen, G., Wille, M., Sorgeloos, P., 2013. Rotifers, *Artemia* and copepods as live feeds for fish larvae in aquaculture, in: Allan, G., Burnell, G. (Eds.), *Advances in aquaculture hatchery technology*. Woodhead Publishing Limited, pp. 157-202.
- Divan, C.L., Schnoes, H.K., 1982. Production of axenic gonyaulax cultures by treatment with antibiotics. *Applied and environmental microbiology*. 44, 250-254.
- Domozych, D.S., Ciancia, M., Fangel, J.U., Mikkelsen, M.D., Ulvskov, P., Willats, W.G.T., 2012. The cell walls of green algae: a journey through evolution and diversity. *Frontiers in Plant Science*. 3, 1-7.
- Dondji, B., Duchon, S., Diabate, A., Herve, J.P., Corbel, V., Hougard, J.M., Santus, R., Schrevel, J., 2005. Assessment of laboratory and field assays of sunlight-induced killing of mosquito larvae by photosensitizers. *Journal of Medical Entomology*. 42, 652-656.
- Dosselli, R., Million, R., Puricelli, L., Tessari, P., Arrigoni, G., Franchin, C., Segalla, A., Teardo, E., Reddi, E., 2012. Molecular targets of antimicrobial photodynamic therapy identified by a proteomic approach. *Journal of Proteomics*. 77, 329-343.
- Doucette, G.J., 1995. Interactions between bacteria and harmful algae: A review. *Natural Toxins*. 3, 65-74.
- Dougherty, T.J., Gomer, C.J., Henderson, B.W., Jori, G., Kessel, D., Korbely, M., Moan, J., Peng, Q., 1998. Photodynamic Therapy. *Journal of the National Cancer Institute*. 90, 889-905.

- Douillet, P.A., 2000. Bacterial additives that consistently enhance rotifer growth under synxenic culture conditions: 1. Evaluation of commercial products and pure isolates. *Aquaculture*. 182, 249-260.
- Drabkova, M., Marsalek, B., Admiraal, W., 2007. Photodynamic therapy against cyanobacteria. *Environmental Toxicology*. 22, 112-115.
- Drinkwater, L.E., Clegg, J.S., 1991. Experimental biology of cyst diapause, in: Sorgeloos, P., Trotman, C.N.A., ,, Browne, R.A. (Eds.), *Artemia biology*. CRC Press, Boca Raton, pp. 93-117.
- Duff, D.C.B., Bruce, D.L., Antia, N.J., 1966. Antibacterial activity of marine planktonic algae. *Canadian Journal of Microbiology*. 12, 877-&.
- Dunn, O.J., 1964. Multiple comparisons using rank sums. *Technometrics*. 6, 241-252.
- Dunnett, C.W., 1980. Pairwise multiple comparisons in the unequal variance case. *Journal of the American Statistical Association*. 75, 796-800.
- Egidius, E., Wiik, R., Andersen, K., Hoff, K., Hjeltnes, B., 1986. *Vibrio salmonicida* sp. nov., a new fish pathogen. *International Journal of Systematic and Evolutionary Microbiology*. 36, 518-520.
- Egles, C., Schastak, S., Ziganshyna, S., Gitter, B., Wiedemann, P., Claudepierre, T., 2010. Efficient photodynamic therapy against Gram-positive and Gram-negative bacteria using THPTS, a cationic photosensitizer excited by infrared wavelength. *PLoS ONE*. 5, e11674.
- El-Magsodi, M.O., Bossier, P., Sorgeloos, P., Van Stappen, G., 2014. Hatching and nutritional quality of *Artemia* cysts progressively deteriorates as a function of increased exposure to hydration/dehydration cycles. *Aquaculture International*. 22, 1515-1532.
- El-Tayeb, T.A., El-Aziz, N.M.A., Awad, H.H., 2013. A study on the dynamics of *Aedes caspius* larval uptake and release of novel haematoporphyrin. *African Entomology*. 21, 15-23.
- Epe, B., 1991. Genotoxicity of singlet oxygen. *Chemico-Biological Interactions*. 80, 239-260.
- Ergaieg, K., Seux, R., 2009. A comparative study of the photoinactivation of bacteria by meso-substituted cationic porphyrin, rose bengal and methylene blue. *Desalination*. 246, 353-362.
- Erzinger, G.S., Wohllebe, S., Vollrath, F., Souza, S.C., Richter, P., Lebert, M., Hader, D.P., 2011. Optimizing conditions for the use of chlorophyll derivatives for photodynamic control of parasites in aquatic ecosystems. *Parasitology Research*. 109, 781-786.
- Espinosa, E.P., Perrigault, M., Ward, J.E., Shumway, S.E., Allam, B., 2010. Microalgal cell surface carbohydrates as recognition sites for particle sorting in suspension-feeding bivalves. *The Biological Bulletin*. 218, 75-86.
- Espinoza-Valles, I., Vora, G.J., Lin, B., Leekitcharoenphon, P., Gonzalez-Castillo, A., Ussery, D., Høj, L., Gomez-Gil, B., 2015. Unique and conserved genome regions

- in *Vibrio harveyi* and related species in comparison with the shrimp pathogen *Vibrio harveyi* CAIM 1792. *Microbiology*. 161, 1762-1779.
- European Union, 1989. Council Directive 89/107/EEC: Food additives authorized for use in foodstuffs intended for human consumption., Geneve.
- Evans, A.S., 1976. Causation and Disease: The Henle-Koch Postulates Revisited. *The Yale Journal of Biology and Medicine*. 49, 175-195.
- Fabris, C., Soncin, M., Jori, G., Habluetzel, A., Lucantoni, L., Sawadogo, S., Guidolin, L., Coppellotti, O., 2012. Effects of a new photoactivatable cationic porphyrin on ciliated protozoa and branchiopod crustaceans, potential components of freshwater ecosystems polluted by pathogenic agents and their vectors. *Photochemical and Photobiological Sciences*. 11, 294-301.
- FAO, 2007. Improving *Penaeus monodon* hatchery practices. Manual based on experience in India. *FAO Fisheries Technical Paper* No. 446, 101.
- FAO, 2012. The state of world fisheries and aquaculture, Rome, pp. 1-209.
- FAO, 2013. CWP handbook of fishery statistical standards - section j: Aquaculture. in: UN), F.a.A.O.o.t.U.N.F.-. (Ed.). Food and Agriculture Organization of the United Nations (FAO- UN), Rome.
- FAO, 2016. The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all, Rome, pp. 1-200.
- Feese, E., Sadeghifar, H., Gracz, H.S., Argyropoulos, D.S., Ghiladi, R.A., 2011. Photobactericidal porphyrin-cellulose nanocrystals: Synthesis, characterization, and antimicrobial properties. *Biomacromolecules*. 12, 3528-3539.
- Felgentrager, A., Gonzales, F.P., Maisch, T., Baumler, W., 2013. Ion-induced stacking of photosensitizer molecules can remarkably affect the luminescence detection of singlet oxygen in *Candida albicans* cells. *Journal of Biomedical Optics*. 18, 8.
- Fernandes, B.D., Mota, A., Teixeira, J.A., Vicente, A.A., 2015. Continuous cultivation of photosynthetic microorganisms: Approaches, applications and future trends. *Biotechnology Advances*. 33, 1228-1245.
- Fernandes, S.O., Kulkarni, S.S., Shirodkar, R.R., Karekar, S.V., Kumar, R.P., Sreepada, R.A., Vogelsang, C., Bharathi, P.A.L., 2010. Water quality and bacteriology in an aquaculture facility equipped with a new aeration system. *Environmental Monitoring and Assessment*. 164, 81-92.
- Finkel, Z.V., Follows, M.J., Liefer, J.D., Brown, C.M., Benner, I., Irwin, A.J., 2016. Phylogenetic diversity in the macromolecular composition of microalgae. *Plos One*. 11, 16.
- Floyd, R.A., Schneider, J.E., Dittmer, D.R., 2004. Methylene blue photoinactivation of RNA viruses. *Antiviral Research*. 61, 141-151.
- Foote, C.S., 1991. Definition of type-i and type-ii photosensitized oxidation. *Photochemistry and Photobiology*. 54, 659-659.
- Fox, J., 2005. The R commander: A basic-statistics graphical user interface to R. *Journal of Statistical Software*. 14, 42.

- Fox, J., 2007. Extending the R Commander by "plug in" packages. *R News*. 3, 46-52.
- Fredricks, D.N., Relman, D.A., 1996. Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical Microbiology Reviews*. 9, 18-33.
- Friedrich, C.L., Moyles, D., Beveridge, T.J., Hancock, R.E.W., 2000. Antibacterial action of structurally diverse cationic peptides on Gram-positive bacteria. *Antimicrobial Agents and Chemotherapy*. 44, 2086-2092.
- Fry, F., 1947. Effect of the environment on animal activity. University of Toronto Press, Toronto,
- Fukuda, Y., Nguyen, H.D., Furuhashi, M., Nakai, T., 1996. Mass mortality of cultured sevenband grouper, *Epinephelus septemfasciatus*, associated with viral nervous necrosis. *Fish Pathology*. 31, 165-170.
- Gasulla, F., Guéra, A., Barreno, E., 2010. A simple and rapid method for isolating lichen photobionts. *Symbiosis*. 51, 175-179.
- Geldin, F., Volkman, J.K., Largeau, C., Derenne, S., Damste, J.S.S., De Leeuw, J.W., 1999. Distribution of aliphatic, non-hydrolyzable biopolymers in marine microalgae. *Organic Geochemistry*. 30, 147-159.
- Geurden, I., Borchert, P., Balasubramanian, M.N., Schrama, J.W., Dupont-Nivet, M., Quillet, E., Kaushik, S.J., Panserat, S., Médale, F., 2013. The positive impact of the early-feeding of a plant-based diet on its future acceptance and utilisation in Rainbow trout. *PLOS ONE*. 8, e83162.
- Giménez, G., Padrós, F., Roque, A., Estévez, A., Furones, D., 2006. Bacterial load reduction of live prey for fish larval feeding using Ox-Aquaculture®. *Aquaculture Research*. 37, 1130-1139.
- Girotti, A.W., 2001. Photosensitized oxidation of membrane lipids: reaction pathways, cytotoxic effects, and cytoprotective mechanisms. *Journal of Photochemistry and Photobiology B: Biology*. 63, 103-113.
- Girotti, A.W., Kriska, T., 2004. Role of lipid hydroperoxides in photo-oxidative stress signaling. *Antioxidants and Redox Signaling*. 6, 301-310.
- Glaeser, J., Nuss, A.M., Berghoff, B.A., Klug, G., 2011. Singlet oxygen stress in microorganisms, in: Robert, K.P. (Ed.), *Advances in Microbial Physiology*. Academic Press, pp. 141-173.
- Gomez-Gil, B., Roque, A., Velasco-Blanco, G., 2002. Culture of *Vibrio alginolyticus* C7b, a potential probiotic bacterium, with the microalga *Chaetoceros muelleri*. *Aquaculture*. 211, 43-48.
- GomezGil-Rs, B., Abreu-Grobois, F.A., Romero-Jarero, J., de los Herrera-Vega, M., 1994. Chemical Disinfection of *Artemia* Nauplii. *Journal of the World Aquaculture Society*. 25, 579-583.
- Goulden, E.F., Hall, M.R., Bourne, D.G., Pereg, L.L., Høj, L., 2012. Pathogenicity and infection cycle of *Vibrio owensii* in larviculture of the ornate spiny lobster (*Panulirus ornatus*). *Applied and Environmental Microbiology*. 78, 2841-2849.

- Grinholc, M., Nakonieczna, J., Fila, G., Taraszkievicz, A., Kawiak, A., Szewczyk, G., Sarna, T., Lilge, L., Bielawski, K.P., 2015. Antimicrobial photodynamic therapy with fulleropyrrolidine: photoinactivation mechanism of *Staphylococcus aureus*, in vitro and in vivo studies. *Applied Microbiology and Biotechnology*. 99, 4031-4043.
- Gsponer, N.S., Spesia, M.B., Durantini, E.N., 2015. Effects of divalent cations, EDTA and chitosan on the uptake and photoinactivation of *Escherichia coli* mediated by cationic and anionic porphyrins. *Photodiagnosis and Photodynamic Therapy*. 12, 67-75.
- Guedes, A.C., Meireles, L.A., Amaro, H.M., Malcata, F.X., 2010. Changes in lipid class and fatty acid composition of cultures of *Pavlova lutheri*, in response to light intensity. *Journal of the American Oil Chemists Society*. 87, 791-801.
- Gügi, B., Le Costaouee, T., Burel, C., Lerouge, P., Helbert, W., Bardor, M., 2015. Diatom-specific oligosaccharides and polysaccharide structures help to unravel biosynthetic capabilities in diatoms. *Marine Drugs*. 13, 5993-6018.
- Guillard, R.R.L., 1975. Culture of phytoplankton for feeding marine invertebrates, in: Smith, W.L., Chanley, M.H. (Eds.), *Culture of Marine Invertebrate Animals*. Plenum press, New York, pp. 29-60.
- Guillard, R.R.L., Hargraves, P.E., 1993. *Stichochrysis immobilis* is a diatom, not a chrysophyte. *Phycologia*. 32, 234-236.
- Hadjur, C., Lange, N., Rebstein, J., Monnier, P., van den Bergh, H., Wagnières, G., 1998. Spectroscopic studies of photobleaching and photoproduct formation of meta(tetrahydroxyphenyl)chlorin (m-THPC) used in photodynamic therapy. The production of singlet oxygen by m-THPC. *Journal of Photochemistry and Photobiology B: Biology*. 45, 170-178.
- Halet, D., Defoirdt, T., Van Damme, P., Vervaeren, H., Forrez, I., Van de Wiele, T., Boon, N., Sorgeloos, P., Bossier, P., Verstraete, W., 2007. Poly-beta-hydroxybutyrate-accumulating bacteria protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbellii*. *FEMS Microbiology Ecology*. 60, 363-369.
- Hall, M.R., Kenway, M., Salmon, M., Francis, D., Goulden, E.F., Høj, L., 2013. Palinurid lobster larval rearing for closed-cycle hatchery production, in: Allan, G., Burnell, G. (Eds.), *Advances in Aquaculture Hatchery Technology*. Woodhead Publishing Limited, pp. 289-328.
- Hamblin, M.R., Hasan, T., 2004. Photodynamic therapy: a new antimicrobial approach to infectious disease? *Photochemical and Photobiological Sciences*. 3, 436-450.
- Han, F., Walker, R.D., Janes, M.E., Prinyawiwatkul, W., Ge, B., 2007. Antimicrobial susceptibilities of *vibrio parahaemolyticus* and *vibrio vulnificus* isolates from Louisiana gulf and retail raw oysters. *Applied and Environmental Microbiology*. 73, 7096-7098.
- Harris, F., Pierpoint, L., 2012. Photodynamic therapy based on 5-aminolevulinic acid and its use as an antimicrobial Agent. *Medicinal Research Reviews*. 32, 1292-1327.

- Harris, L., 1993. An investigation into the virulence of strains of *Vibrio harveyi* pathogenic to larvae of the black tiger prawn *Penaeus monodon*. James Cook University, Townsville.
- Harris, L.J., Owens, L., 1999. Production of exotoxins by two luminous *Vibrio harveyi* strains known to be primary pathogens of *Penaeus monodon* larvae. *Diseases of Aquatic Organisms*. 38, 11-22.
- Hauton, C., Hudspith, M., Gunton, L., 2015. Future prospects for prophylactic immune stimulation in crustacean aquaculture - the need for improved metadata to address immune system complexity. *Developmental and Comparative Immunology*. 48, 360-368.
- Henley, W.J., Hironaka, J.L., Guillou, L., Buchheim, M.A., Buchheim, J.A., Fawley, M.W., Fawley, K.P., 2004. Phylogenetic analysis of the 'Nannochloris-like' algae and diagnoses of *Picochlorum oklahomensis* gen. et sp. nov. (*Trebouxiophyceae*, *Chlorophyta*). *Phycologia*. 43, 641-652.
- Hibberd, D.J., 1981. Notes on the taxonomy and nomenclature of the algal classes Eustigmatophyceae and Tribophyceae (synonym Xanthophyceae). *Botanical Journal of the Linnean Society*. 82, 93-119.
- Hoffmann, M., Monday, S.R., Fischer, M., Brown, E.W., 2012. Genetic and phylogenetic evidence for misidentification of *Vibrio* species within the Harveyi clade. *Letters in Applied Microbiology*. 54, 160-165.
- Høj, L., Bourne, D.G., Hall, M.R., 2009. Localization, abundance and community structure of bacteria associated with *Artemia*: Effects of nauplii enrichment and antimicrobial treatment. *Aquaculture*. 293, 278-285.
- Hollander, M., Wolfe, D.A., 1973. *Nonparametric statistical methods*. John Wiley & Sons, New York.
- Hori, T., Norris, R.E., Chihara, M., 1986. Studies on the ultrastructure and taxonomy of the genus *Tetraselmis* (*prasinophyceae*) III. Subgenus *parviselmis*. *Botanical Magazine Tokyo*. 99, 123-135.
- Hoshaw, R., Rosowski, J., 1973. *Methods for microscopic algae*. Handbook of Phycological Methods. Cambridge University Press, New York, 53-67.
- Igarashi, M.A., Sugita, H., Deguchi, Y., 1989. Microflora associated with eggs and nauplii of *Artemia salina*. *Nippon Suisan Gakkaishi*. 55, 2045-2045.
- Interaminense, J.A., Calazans, N.F., do Valle, B.C., Vogeley, J.L., Peixoto, S., Soares, R., Lima, J.V., 2014. *Vibrio* spp. control at brine shrimp, *Artemia*, hatching and enrichment. *Journal of the World Aquaculture Society*. 45, 65-74.
- Islam, M.A., Magnusson, M., Brown, R.J., Ayoko, G.A., Nabi, M.N., Heimann, K., 2013. Microalgal species selection for biodiesel production based on fuel properties derived from fatty acid profiles. *Energies*. 6, 5676-5702.
- Jancula, D., Drabkova, M., Cerny, J., Karaskova, M., Korinkova, R., Rakusan, J., Marsalek, B., 2008. Algicidal activity of phthalocyanines - Screening of 31 compounds. *Environmental Toxicology*. 23, 218-223.

- Jarvi, M.T., Niedre, M.J., Patterson, M.S., Wilson, B.C., 2011. The influence of oxygen depletion and photosensitizer triplet-state dynamics during photodynamic therapy on accurate singlet oxygen luminescence monitoring and analysis of treatment dose response. *Photochemistry and Photobiology*. 87, 223-234.
- Jemli, M., Alouini, Z., Sabbahi, S., Gueddari, M., 2002. Destruction of fecal bacteria in wastewater by three photosensitizers. *Journal of Environmental Monitoring*. 4, 511-516.
- Jensen, M.A., Ritar, A.J., Burke, C., Ward, L.R., 2011. Seawater ozonation and formalin disinfection for the larval culture of eastern rock lobster, *Jasus (Sagmariasus) verreauxi*, phyllosoma. *Aquaculture*. 318, 213-222.
- Jiang, X.P., Chai, T.J., 1996. Survival of *Vibrio parahaemolyticus* at low temperatures under starvation conditions and subsequent resuscitation of viable, nonculturable cells. *Applied and Environmental Microbiology*. 62, 1300-1305.
- Jithendran, K.P., Poornima, M., Balasubramanian, C.P., Kulasekarapandian, S., 2010. Diseases of mud crabs (*Scylla* spp.): an overview. *Indian Journal of Fisheries*. 57, 55-63.
- Jöborn, A., Olsson, J.C., Westerdahl, A., Conway, P.L., Kjelleberg, S., 1997. Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. *Journal of Fish Diseases*. 20, 383-392.
- Johnston, M.D., Brown, M.H., 2002. An investigation into the changed physiological state of *Vibrio* bacteria as a survival mechanism in response to cold temperatures and studies on their sensitivity to heating and freezing. *Journal of Applied Microbiology*. 92, 1066-1077.
- Joint, I., Tait, K., Callow, M.E., Callow, J.A., Milton, D., Williams, P., Camara, M., 2002. Cell-to-cell communication across the prokaryote-eukaryote boundary. *Science*. 298, 1207-1207.
- Jori, G., Brown, S.B., 2004. Photosensitized inactivation of microorganisms. *Photochemical and Photobiological Sciences*. 3, 403-405.
- Jori, G., Fabris, C., Soncin, M., Ferro, S., Coppellotti, O., Dei, D., Fantetti, L., Chiti, G., Roneucci, G., 2006. Photodynamic therapy in the treatment of microbial infections: Basic principles and perspective applications. *Lasers in Surgery and Medicine*. 38, 468-481.
- Jori, G., Coppellotti, O., 2007. Inactivation of pathogenic microorganisms by photodynamic techniques: Mechanistic aspects and perspective applications. *Current Medicinal Chemistry: Anti-Infective Agents*. 6, 119-131.
- Jori, G., Magaraggia, M., Fabris, C., Soncin, M., Camerin, M., Tallandini, L., Coppellotti, O., Guidolin, L., 2011. Photodynamic inactivation of microbial pathogens: Disinfection of water and prevention of water-borne diseases. *Journal of Environmental Pathology Toxicology and Oncology*. 30, 261-271.

- Jorquera, M.A., Valencia, G., Eguchi, M., Katayose, M., Riquelme, C., 2002. Disinfection of seawater for hatchery aquaculture systems using electrolytic water treatment. *Aquaculture*. 207, 213-224.
- Kader, M.A., Koshio, S., 2012. Effect of composite mixture of seafood by-products and soybean proteins in replacement of fishmeal on the performance of red sea bream, *Pagrus major*. *Aquaculture*. 368, 95-102.
- Kanyal, P., Roshan, R.P.M., Danish, M., Anita, Khati, A., and Chauhan, R.S., 2016. Antimicrobial photodynamic therapy and its applicability in aquaculture systems and aquatic animal health management: An overview. *Journal of Applied and Natural Science*. 8, 506-514.
- Karunasagar, I., Pai, R., Malathi, G.R., 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture*. 128, 203-209.
- Kazamia, E., Czesnick, H., Nguyen, T.T., Croft, M.T., Sherwood, E., Sasso, S., Hodson, S.J., Warren, M.J., Smith, A.G., 2012. Mutualistic interactions between vitamin B12 - dependent algae and heterotrophic bacteria exhibit regulation. *Environmental microbiology*. 14, 1466-1476.
- Kessel, D., Smith, K., 1989. Photosensitization with derivatives of chlorophyll. *Photochemistry and Photobiology*. 49, 157-160.
- Kim, B.-H., Ramanan, R., Cho, D.-H., Oh, H.-M., Kim, H.-S., 2014. Role of Rhizobium, a plant growth promoting bacterium, in enhancing algal biomass through mutualistic interaction. *Biomass and Bioenergy*. 69, 95-105.
- Kim, J.Y., Lee, J.-L., 2017. Correlation of total bacterial and *Vibrio* spp. populations between fish and water in the aquaculture system. *Frontiers in Marine Science*. 4.
- Kim, S.Y., Kwon, O.J., Park, J.W., 2001. Inactivation of catalase and superoxide dismutase by singlet oxygen derived from photoactivated dye. *Biochimie*. 83, 437-444.
- Knuckey, R.M., Brown, M.R., Barrett, S.M., Hallegraef, G.M., 2002. Isolation of new nanoplanktonic diatom strains and their evaluation as diets for juvenile Pacific oysters (*Crassostrea gigas*). *Aquaculture*. 211, 253-274.
- Kogure, K., Simidu, U., Taga, N., 1979. Effect of *Skeletonema costatum* (Grev) cleve on the growth of marine bacteria. *Journal of Experimental Marine Biology and Ecology*. 36, 201-215.
- Komagoe, K., Kato, H., Inoue, T., Katsu, T., 2011. Continuous real-time monitoring of cationic porphyrin-induced photodynamic inactivation of bacterial membrane functions using electrochemical sensors. *Photochemical & Photobiological Sciences*. 10, 1181-1188.
- Komerik, N., Wilson, M., Poole, S., 2000. The effect of photodynamic action on two virulence factors of Gram-negative bacteria. *Photochemistry and Photobiology*. 72, 676-680.
- Konopka, K., Goslinski, T., 2007. Photodynamic therapy in dentistry. *Journal of Dental Research*. 86, 694-707.

- Korkea-aho, T.L., Papadopoulou, A., Heikkinen, J., von Wright, A., Adams, A., Austin, B., Thompson, K.D., 2012. *Pseudomonas* M162 confers protection against rainbow trout fry syndrome by stimulating immunity. *Journal of applied microbiology*. 113, 24-35.
- Kouba, A., Hamáčková, J., Buřič, M., Polícar, T., Kozák, P., 2011. Use of three forms of decapsulated *Artemia* cysts as food for juvenile noble crayfish (*Astacus astacus*). *Czech Journal of Animal Science*. 56, 114–118.
- Krasnovsky, A.A., Jr., 1998. Singlet molecular oxygen in photobiochemical systems: IR phosphorescence studies. *Membrane and Cell Biology*. 12, 665-690.
- Krouit, M., Granet, R., Branland, P., Verneuil, B., Krausz, P., 2006. New photoantimicrobial films composed of porphyrinated lipophilic cellulose esters. *Bioorganic and Medicinal Chemistry Letters*. 16, 1651-1655.
- Krouit, M., Granet, R., Krausz, P., 2008. Photobactericidal plastic films based on cellulose esterified by chloroacetate and a cationic porphyrin. *Bioorganic and Medicinal Chemistry*. 16, 10091-10097.
- Kuban, F.D., Wilkenfeld, J.S., Lawrence, A.L., 1983. Survival and growth of *Penaeus setiferus* L. and *Penaeus aztecus* Ives larvae fed *Artemia* beginning at the protozoa-two substage versus the mysis-one substage. *Journal of the World Mariculture Society*. 14, 38-48.
- Kumaresan, V., Palanisamy, R., Pasupuleti, M., Arockiaraj, J., 2016. Impacts of environmental and biological stressors on immune system of *Macrobrachium rosenbergii*. *Reviews in Aquaculture*, n/a-n/a.
- Kuo, R.C., Lin, S., 2013. Ectobiotic and endobiotic bacteria associated with *Eutreptiella* sp. isolated from Long Island Sound. *Protist*. 164, 60-74.
- Kuznetsova, N.A., Makarov, D.A., Yuzhakova, O.A., Solovieva, L.I., Kaliya, O.L., 2010. Study on the photostability of water-soluble Zn(II) and Al(III) phthalocyanines in aqueous solution. *Journal of Porphyrins and Phthalocyanines*. 14, 968-974.
- Kuznetsova, N.A., Yuzhakova, O.A., Strakhovskaya, M.G., Shumarina, A.O., Kozlov, A.S., Krasnovsky, A.A., Kaliya, O.L., 2011. New heterogeneous photosensitizers with phthalocyanine molecules covalently linked to aminopropyl silica gel. *Journal of Porphyrins and Phthalocyanines*. 15, 718-726.
- Lakshmi, B., Viswanath, B., Sai Gopal, D.V.R., 2013. Probiotics as antiviral agents in shrimp aquaculture. *Journal of pathogens*. 2013, 424123.
- Lam, C., Stang, A., Harder, T., 2008. Planktonic bacteria and fungi are selectively eliminated by exposure to marine macroalgae in close proximity. *FEMS Microbiology Ecology*. 63, 283-291.
- Laranja, J.L.Q., Ludeyese-Pascual, G.L., Amar, E.C., Sorgeloos, P., Bossier, P., De Schryver, P., 2014. Poly-beta-hydroxybutyrate (PHB) accumulating *Bacillus* spp. improve the survival, growth and robustness of *Penaeus monodon* (Fabricius, 1798) postlarvae. *Veterinary Microbiology*. 173, 310-317.
- Lau, M.K., 2013. DTK: Dunnett-Tukey-Kramer pairwise multiple comparison test adjusted for unequal variances and unequal sample sizes, R package version 3.5.

- Lavens, P., Tackaert, W., Sorgeloos, P., 1986. International study on *Artemia*. XLI. Influence of culture conditions and specific diapause deactivation methods on the hatchability of *Artemia* cysts produced in a standard culture system. Marine Ecology Progress Series. 31, 197-203.
- Lavens, P., Sorgeloos, P., 1987. The cryptobiotic state of *Artemia* cysts, its diapause deactivation and hatching: a review. *Artemia research and its applications*. 3, 27-63.
- Lavens, P., Sorgeloos, P., 1996. Manual on the production and use of live food for aquaculture. Food and Agriculture Organization (FAO). 9251039348.
- Lazado, C.C., Caipang, C.M., Brinchmann, M.F., Kiron, V., 2011. In vitro adherence of two candidate probiotics from Atlantic cod and their interference with the adhesion of two pathogenic bacteria. *Veterinary microbiology*. 148, 252-259.
- Lazzeri, D., Rovera, M., Pascual, L., Durantini, E., 2004. Photodynamic studies and photoinactivation of *Escherichia coli* using meso-substituted cationic porphyrin derivatives with asymmetric charge distribution. *Photochemistry and Photobiology*. 80, 286 - 293.
- Lee, C.-S., O'Bryen, P.J., 2001. Biosecurity in aquaculture production systems: Exclusion of pathogens and other undesirables,
- Lee, Y., Baron, E.D., 2011. Photodynamic therapy: current evidence and applications in dermatology. *Seminars in cutaneous medicine and surgery*. 30, 199-209.
- Léger, P., Bengtson, D.A., Sorgeloos, P., Simpson, K.L., Beck, A.D., 1987. The nutritional value of *Artemia*: a review, in: Sorgeloos, P., Bengtson, D.A., Declair, W., Jaspers, E. (Eds.), *Artemia Research and its Applications. Ecology, Culturing, Use in Aquaculture*. Universa Press, Wetteren, Belgium., pp. 357-372.
- Lemmermann, E., 1898. Der grosse Waterneverstorfer Binnensee. Eine biologische Studie. *Forschungsberichte aus der Biologischen Station zu Plön*. 6, 166-205.
- Likodimos, V., Dionysiou, D.D., Falaras, P., 2010. CLEAN WATER: water detoxification using innovative photocatalysts. *Reviews in Environmental Science and Biotechnology*. 9, 87-94.
- Lio-Po, G.D., Leano, E.M., Penaranda, M.M.D., Villa-Franco, A.U., Sombito, C.D., Guanzon, N.G., 2005. Anti-luminous *vibrio* factors associated with the 'green water' grow-out culture of the tiger shrimp *Penaeus monodon*. *Aquaculture*. 250, 1-7.
- Liu, J., Chen, F., 2016. Biology and industrial applications of *Chlorella*: Advances and prospects. *Advances in Biochemical, Engineering/Biotechnology*. 153, 1-35.
- Lopez-Torres, M.A., Lizarraga-Partida, M.L., 2001. Bacteria isolated on TCBS media associated with hatched *Artemia* cysts of commercial brands. *Aquaculture*. 194, 11-20.
- Lozano-Leon, A., Torres, J., Osorio, C.R., Martinez-Urtaza, J., 2003. Identification of tdh-positive *Vibrio parahaemolyticus* from an outbreak associated with raw oyster consumption in Spain. *FEMS Microbiology Letters*. 226, 281-284.

- Lucantoni, L., Magaraggia, M., Lupidi, G., Ouedraogo, R.K., Coppellotti, O., Esposito, F., Fabris, C., Jori, G., Habluetzel, A., 2011. Novel, meso-substituted cationic porphyrin molecule for photo-mediated larval control of the dengue vector *Aedes aegypti*. PLOS Neglected Tropical Diseases. 5, e1434.
- Ma, D., Hu, Y., Wang, J., Ye, S., Li, A., 2006. Effects of antibacterials use in aquaculture on biogeochemical processes in marine sediment. The Science of the Total Environment. 367, 273-277.
- Macey, B.M., Coyne, V.E., 2006. Colonization of the gastrointestinal tract of the farmed South African abalone *Haliotis midae* by the probionts *Vibrio midae* SY9, *Cryptococcus* sp. SS1, and *Debaryomyces hanseni* AY1. Marine biotechnology. 8, 246-259.
- Maclean, M., Macgregor, S.J., Anderson, J.G., Woolsey, G.A., 2008. The role of oxygen in the visible-light inactivation of *Staphylococcus aureus*. Journal of photochemistry and photobiology B: Biology. 92, 180-184.
- MacRae, T.H., 2003. Molecular chaperones, stress resistance and development in *Artemia franciscana*. Seminars in Cell and Developmental Biology. 14, 251-258.
- MacRae, T.H., 2005. Diapause: diverse states of developmental and metabolic arrest. Journal of Biological Research. 3, 3-14.
- Magaraggia, M., Faccenda, F., Gandolfi, A., Jori, G., 2006. Treatment of microbiologically polluted aquaculture waters by a novel photochemical technique of potentially low environmental impact. Journal of Environmental Monitoring. 8, 923-931.
- Maisch, T., Szeimies, R.-M., Jori, G., Abels, C., 2004. Antibacterial photodynamic therapy in dermatology. Photochemical and Photobiological Sciences. 3, 907-917.
- Maisch, T., Bosl, C., Szeimies, R.-M., Lehn, N., Abels, C., 2005. Photodynamic effects of novel XF porphyrin derivatives on prokaryotic and eukaryotic cells. Antimicrobial Agents and Chemotherapy. 49, 1542-1552.
- Maisch, T., 2007. Anti-microbial photodynamic therapy: useful in the future? Lasers in Medical Science. 22, 83-91.
- Maisch, T., 2009. A new strategy to destroy antibiotic resistant microorganisms: antimicrobial photodynamic treatment. Mini-Reviews in Medicinal Chemistry. 9, 974-983.
- Maisch, T., Spannberger, F., Regensburger, J., Felgentrager, A., Baumler, W., 2012. Fast and effective: intense pulse light photodynamic inactivation of bacteria. Journal of industrial microbiology & biotechnology. 39, 1013-1021.
- Maisch, T., 2015a. Strategies to optimize photosensitizers for photodynamic inactivation of bacteria. Journal of Photochemistry and Photobiology B: Biology. 150, 2-10.
- Maisch, T., 2015b. Resistance in antimicrobial photodynamic inactivation of bacteria. Photochemical and Photobiological Sciences. 14, 1518-1526.
- Makridis, P., Fjellheim, A.J., Skjermo, J., Vadstein, O., 2000. Control of the bacterial flora of *Brachionus plicatilis* and *Artemia franciscana* by incubation in bacterial suspensions. Aquaculture. 185, 207-218.

- Makridis, P., Bergh, Ø., Skjermo, J., Vadstein, O., 2001. Addition of bacteria bioencapsulated in *Artemia metanauplii* to a rearing system for halibut larvae. *Aquaculture International*. 9, 225-235.
- Malara, D., Høj, L., Heimann, K., Citarrella, G., Oelgemöller, M., 2017a. Capacity of cationic and anionic porphyrins to inactivate the potential aquaculture pathogen *Vibrio campbellii*. *Aquaculture*. 473, 228-236.
- Malara, D., Mielke, C., Oelgemöller, M., Senge, M.O., Heimann, K., 2017b. Sustainable water treatment in aquaculture – photolysis and photodynamic therapy for the inactivation of *Vibrio* species. *Aquaculture Research*. 48, 2954–2962.
- Malik, Z., Hanania, J., Nitzan, Y., 1990. New trends in photobiology bactericidal effects of photoactivated porphyrins — An alternative approach to antimicrobial drugs. *Journal of Photochemistry and Photobiology B: Biology*. 5, 281-293.
- Manjón, F., Villén, L., García-Fresnadillo, D., Orellana, G., 2008. On the factors influencing the performance of solar reactors for water disinfection with photosensitized singlet oxygen. *Environmental Science and Technology*. 42, 301-307.
- Manjón, F., Santana-Magana, M., Garcia-Fresnadillo, D., Orellana, G., 2010. Singlet oxygen sensitizing materials based on porous silicone: photochemical characterization, effect of dye reloading and application to water disinfection with solar reactors. *Photochemical and Photobiological Sciences*. 9, 838-845.
- Marciel, L., Teles, L., Moreira, B., Pacheco, M., Lourenco, L.M., Neves, M.G., Tome, J.P., Faustino, M.A., Almeida, A., 2017. An effective and potentially safe blood disinfection protocol using tetrapyrrolic photosensitizers. *Future Medicinal Chemistry*. 9, 365-379.
- Martin, G.G., Rubin, N., Swanson, E., 2004. *Vibrio parahaemolyticus* and *V. harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp *Sicyonia ingentis*. *Diseases of Aquatic Organisms*. 60, 21-29.
- Martinez-Fernandez, E., Acosta-Salmon, H., Southgate, P.C., 2006. The nutritional value of seven species of tropical microalgae for black-lip pearl oyster (*Pinctada margaritifera*, L.) larvae. *Aquaculture*. 257, 491-503.
- McCullagh, C., Robertson, P.K., 2006a. Effect of polyethylenimine, a cell permeabilizer, on the photosensitized destruction of algae by methylene blue and nuclear fast red. *Photochemistry and Photobiology*. 82, 1662-1667.
- McCullagh, C., Robertson, P.K.J., 2006b. Photo-dynamic biocidal action of methylene blue and hydrogen peroxide on the cyanobacterium *Synechococcus leopoliensis* under visible light irradiation. *Journal of Photochemistry and Photobiology B-Biology*. 83, 63-68.
- McCullagh, C., Robertson, P.K.J., 2006c. Photosensitized destruction of *Chlorella vulgaris* by methylene blue or nuclear fast red combined with hydrogen peroxide under visible light irradiation. *Environmental Science and Technology*. 40, 2421-2425.
- Meisel, P., Kocher, T., 2005. Photodynamic therapy for periodontal diseases: state of the art. *Journal of Photochemistry and Photobiology B: Biology*. 79, 159-170.

- Merchat, M., Bertolini, G., Giacomini, P., Villanueva, A., Jori, G., 1996a. Meso-substituted cationic porphyrins as efficient photosensitizers of Gram-positive and Gram-negative bacteria. *Journal of Photochemistry and Photobiology B: Biology*. 32, 153-157.
- Merchat, M., Spikes, J.D., Bertolini, G., Jori, G., 1996b. Studies on the mechanism of bacteria photosensitization by meso-substituted cationic porphyrins. *Journal of Photochemistry and Photobiology B: Biology*. 35, 149-157.
- Mesquita, M.Q., Menezes, J., Neves, M., Tome, A.C., Cavaleiro, J.A.S., Cunha, A., Almeida, A., Hackbarth, S., Roder, B., Faustino, M.A.F., 2014. Photodynamic inactivation of bioluminescent *Escherichia coli* by neutral and cationic pyrrolidine-fused chlorins and isobacteriochlorins. *Bioorganic and Medicinal Chemistry Letters*. 24, 808-812.
- Meyer, F.P., 1991. Aquaculture disease and health management. *Journal of animal science*. 69, 4201-4208.
- Michels, S., Schmidt-Erfurth, U., 2001. Photodynamic therapy with verteporfin: a new treatment in ophthalmology. *Seminars in ophthalmology*. 16, 201-206.
- Mitton, D., Ackroyd, R., 2008. A brief overview of photodynamic therapy in Europe. *Photodiagnosis and Photodynamic Therapy*. 5, 103-111.
- Moan, J., 1986. Effect of bleaching of porphyrin sensitizers during photodynamic therapy. *Cancer Letters*. 33, 45-53.
- Moan, J., 1990. On the diffusion length of singlet oxygen in cells and tissues. *Journal of Photochemistry and Photobiology B: Biology*. 6, 343-344.
- Mroz, P., Tegos, G.P., Gali, H., Wharton, T., Sarna, T., Hamblin, M.R., 2007. Photodynamic therapy with fullerenes. *Photochemical and Photobiological Sciences*. 6, 1139-1149.
- Muir, P.R., 1991. Factors affecting the survival of penaeid prawns. James Cook University, Townsville.
- Muller-Breitkreutz, K., Mohr, H., Briviba, K., Sies, H., 1995. Inactivation of viruses by chemically and photochemically generated singlet molecular oxygen. *Journal of photochemistry and photobiology B: Biology*. 30, 63-70.
- Munro, P.D., Henderson, R.J., Barbour, A., Birkbeck, T.H., 1999. Partial decontamination of rotifers with ultraviolet radiation: the effect of changes in the bacterial load and flora of rotifers on mortalities in start-feeding larval turbot. *Aquaculture*. 170, 229-244.
- Murchelano, R.A., Brown, C., 1969. Bacterial flora of some algal foods used for rearing bivalve larvae. *Journal of the Fisheries Board of Canada*. 32, 739-745.
- Muroga, K., Iida, M., Matsumoto, H., Nakai, T., 1986. Detection of *Vibrio anguillarum* from waters. *Bulletin of the Japanese Society of Scientific Fisheries*. 52, 641-647.
- Muroga, K., Tanasomwang, V., Momoyama, K., 1987. *Vibrio anguillarum* infection in tiger puffer (*takifugu rubripes*) fingerlings. *Fish Pathology*. 22, 29-30.

- Muroga, K., 2001. Viral and bacterial diseases of marine fish and shellfish in Japanese hatcheries. *Aquaculture*. 202, 23-44.
- Myre, E., Shaw, R., 2006. The turbidity tube: Simple and accurate measurement of turbidity in the field. Michigan Technological University.
- Nagayama, K., Iwamura, Y., Shibata, T., Hirayama, I., Nakamura, T., 2002. Bactericidal activity of phlorotannins from the brown alga *Ecklonia kurome*. *Journal of Antimicrobial Chemotherapy*. 50, 889-893.
- Nakai, T., Park, S.C., 2002. Bacteriophage therapy of infectious diseases in aquaculture. *Research in Microbiology*. 153, 13-18.
- Nambu, Z., Tanaka, S., Nambu, F., Nakano, M., 2008. Influence of temperature and darkness on embryonic diapause termination in dormant *Artemia* cysts that have never been desiccated. *Journal of Experimental Zoology Part a: Ecological Genetics and Physiology*. 309A, 17-24.
- Nascimento, C.L., Ribeiro, M.S., Sellera, F.P., Dutra, G.H., Simoes, A., Teixeira, C.R., 2015. Comparative study between photodynamic and antibiotic therapies for treatment of footpad dermatitis (bumblefoot) in Magellanic penguins (*Spheniscus magellanicus*). *Photodiagnosis and Photodynamic Therapy*. 12, 36-44.
- Natrah, F.M.I., Bossier, P., Sorgeloos, P., Yusoff, F.M., Defoirdt, T., 2014. Significance of microalgal- bacterial interactions for aquaculture. *Reviews in Aquaculture*. 6, 48-61.
- Ndong, D., Chen, Y.Y., Lin, Y.H., Vaseeharan, B., Chen, J.C., 2007. The immune response of tilapia *Oreochromis mossambicus* and its susceptibility to *Streptococcus iniae* under stress in low and high temperatures. *Fish and Shellfish Immunology*. 22, 686-694.
- Newaj-Fyzul, A., Al-Harbi, A.H., Austin, B., 2014. Review: Developments in the use of probiotics for disease control in aquaculture. *Aquaculture*. 431, 1-11.
- Nicolas, J.L., Corre, S., Cochard, J.C., 2004. Bacterial population association with phytoplankton cultured in a bivalve hatchery. *Microbial ecology*. 48, 400-413.
- Nitzan, Y., Shainberg, B., Malik, Z., 1989. The mechanism of photodynamic inactivation of *Staphylococcus aureus* by deuteroporphyrin. *Current Microbiology*. 19, 265-269.
- Nitzan, Y., Gutterman, M., Malik, Z., Ehrenberg, B., 1992. Inactivation of Gram-negative bacteria by photosensitized porphyrins. *Photochemistry and Photobiology*, 89 - 96.
- Nitzan, Y., Ashkenazi, H., 2001. Photoinactivation of *Acinetobacter baumannii* and *Escherichia coli* B by a cationic hydrophilic porphyrin at various light wavelengths. *Current Microbiology*. 42, 408-414.
- Noctor, G., Foyer, C.H., 1998. Ascorbate and glutathione: Keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology*. 49, 249-279.

- Nunan, L., Lightner, D., Pantoja, C., Gomez-Jimenez, S., 2014. Detection of acute hepatopancreatic necrosis disease (AHPND) in Mexico. *Diseases of Aquatic Organisms*. 111, 81-+.
- Nyman, E.S., Hynninen, P.H., 2004. Research advances in the use of tetrapyrrolic photosensitizers for photodynamic therapy. *Journal of Photochemistry and Photobiology B: Biology*. 73, 1-28.
- O'Connor, A.E., Gallagher, W.M., Byrne, A.T., 2009. Porphyrin and nonporphyrin photosensitizers in oncology: Preclinical and clinical advances in photodynamic therapy. *Photochemistry and Photobiology*. 85, 1053-1074.
- Ochsner, M., 1997. Photophysical and photobiological processes in the photodynamic therapy of tumours. *Journal of Photochemistry and Photobiology B: Biology*. 39, 1-18.
- Olafsen, J.A., 2001. Interactions between fish larvae and bacteria in marine aquaculture. *Aquaculture*. 200, 223-247.
- Olaizola, M., 2003. Commercial development of microalgal biotechnology: from the test tube to the marketplace. *Biomolecular Engineering*. 20, 459-466.
- Oliveira, A., Almeida, A., Carvalho, C., Tome, J., Faustino, M., Neves, M., Tome, A., Cavaleiro, J., Cunha, A., 2009. Porphyrin derivatives as photosensitizers for the inactivation of *Bacillus cereus* endospores. *Journal of Applied Microbiology*.
- Oliveira, J., Castilho, F., Cunha, A., Pereira, M.J., 2012. Bacteriophage therapy as a bacterial control strategy in aquaculture. *Aquaculture International*. 20, 879-910.
- Olsen, A.I., Olsen, Y., Attramadal, Y., Christie, K., Birkbeck, T.H., Skjermo, J., Vadstein, O., 2000. Effects of short term feeding of microalgae on the bacterial flora associated with juvenile *Artemia franciscana*. *Aquaculture*. 190, 11-25.
- Olson, R.J., Chisholm, S.W., 1986. Effects of light and nitrogen limitation on the cell cycle of the dinoflagellate *Amphidinium carteri*. *Journal of Plankton Research*. 8, 785-793.
- Ortner, M.-A., 2009. Photodynamic therapy for cholangiocarcinoma: overview and new developments. *Current Opinion in Gastroenterology*. 25, 472-476.
- Owens, L., Busico-Salcedo, N., 2006. *Vibrio harveyi*: Pretty Problems in Paradise, The Biology of Vibrios. American Society of Microbiology.
- Pandey, A., Lee, D.J., Chisti, Y., Soccol, C.R., 2014. Biofuels from Algae, 978-0-444-59558-4(H); 978-0-444-59582-9(P).
- Pansonato, N., Afonso, M.V.G., Salles, C.A., Boncz, M.A., Paulo, P.L., 2011. Solar disinfection for the post-treatment of greywater by means of a continuous flow reactor. *Water Science and Technology*. 64, 1178-1185.
- Pavani, C., Uchoa, A.F., Oliveira, C.S., Iamamoto, Y., Baptista, M.S., 2009. Effect of zinc insertion and hydrophobicity on the membrane interactions and PDT activity of porphyrin photosensitizers. *Photochemical and Photobiological Sciences*. 8, 233-240.

- Pavinatto, F.J., Gameiro, A.F., Jr., Hidalgo, A.A., Dinelli, L.R., Romualdo, L.L., Batista, A.A., Barbosa Neto, N.M., Ferreira, M., Oliveira, O.N., Jr., 2008. Langmuir and Langmuir-Blodgett (LB) films of tetrapyrridyl metalloporphyrins. *Applied Surface Science*. 254, 5946-5952.
- Payne, M.S., Hall, M.R., Bannister, R., Sly, L., Bourne, D.G., 2006. Microbial diversity within the water column of a larval rearing system for the ornate rock lobster (*Panulirus ornatus*). *Aquaculture*. 258, 80-90.
- Paz-Cristobal, M.P., Royo, D., Rezusta, A., Andres-Ciriano, E., Alejandre, M.C., Meis, J.F., Revillo, M.J., Aspiroz, C., Nonell, S., Gilaberte, Y., 2014. Photodynamic fungicidal efficacy of hypericin and dimethyl methylene blue against azole-resistant *Candida albicans* strains. *Mycoses*. 57, 35-42.
- Peggy, A.R., Francis-Floyd, R., 1996. *Vibrio* infection of Fish. University of Florida Cooperative Extension Service, Institute of Food and Agriculture Sciences, EDIS, Gainesville, Fla.,
- Pellosi, D.S., Batistela, V.R., de Souza, V.R., Scarminio, I.S., Caetano, W., Hioka, N., 2013. Evaluation of the photodynamic activity of xanthene dyes on *Artemia salina* described by chemometric approaches. *Anais Da Academia Brasileira De Ciencias*. 85, 1267-1274.
- Peloi, L.S., Soares, R.R.S., Biondo, C.E.G., Souza, V.R., Hioka, N., Kimura, E., 2008. Photodynamic effect of light-emitting diode light on cell growth inhibition induced by methylene blue. *Journal of Biosciences*. 33, 231-237.
- Pereira, C., Moreirinha, C., Teles, L., Rocha, R.J.M., Calado, R., Romalde, J.L., Nunes, M.L., Almeida, A., 2017. Application of phage therapy during bivalve depuration improves *Escherichia coli* decontamination. *Food Microbiology*. 61, 102-112.
- Pereira Gonzales, F., Maisch, T., 2012. Photodynamic inactivation for controlling *Candida albicans* infections. *Fungal Biology*. 116, 1-10.
- Philippova, T.O., Galkin, B.N., Zinchenko, O.Y., Rusakova, M.Y., Ivanitsa, V.A., Zhilina, Z.I., Vodzinskii, S.V., Ishkov, Y.V., 2003. The antimicrobial properties of new synthetic porphyrins. *Journal of Porphyrins and Phthalocyanines*. 7, 755-760.
- Pintado, J., Planas, M., Makridis, P., 2014. Live feeds: microbial assemblages, probiotics and prebiotics, in: Merrifield, D., Ringo, E. (Eds.), *Aquaculture nutrition: gut health, probiotics and prebiotics*. John Wiley & Sons Ltd, the Atrium, Southern Gate, Chichester, West Sussex, UK, pp. 419-442.
- Pizzutto, M., Hirst, R.G., 1995. Classification of isolates of *Vibrio-harveyi* virulent to *Penaeus-monodon* larvae by protein profile analysis and m13 DNA-fingerprinting. *Diseases of Aquatic Organisms*. 21, 61-68.
- Pohl, J., Röder, B., 2015. Photodynamic inactivation of phototrophic microorganisms. *Photonics and Lasers in Medicine*. 4, 340-341.
- Pohl, J., Saltsman, I., Mahammed, A., Gross, Z., Roder, B., 2015. Inhibition of green algae growth by corrole-based photosensitizers. *Journal of Applied Microbiology*. 118, 305-312.

- Pohlert, T., 2014. The pairwise multiple comparison of mean ranks package (PMCMR). in: package, R. (Ed.).
- Porra, R.J., 2011. Appendix A: A proven simultaneous equation assay for chlorophylls a and b using aqueous acetone and similar assays for recalcitrant algae, in: Roy, S., Llewellyn, C.A., Egeland, E.S., Johnsen, G. (Eds.), *Phytoplankton Pigments: Characterization, chemotaxonomy and applications in oceanography*. Cambridge University Press, New York, pp. 366-374.
- Pörtner, H.O., Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science*. 315, 95-97.
- Prado, S., Romalde, J.L., Barja, J.L., 2010. Review of probiotics for use in bivalve hatcheries. *Veterinary Microbiology*. 145, 187-197.
- Puente, M.E., Vegavillasante, F., Holguin, G., Bashan, Y., 1992. Susceptibility of the brine shrimp *artemia* and its pathogen *vibrio-parahaemolyticus* to chlorine dioxide in contaminated sea-water. *Journal of Applied Bacteriology*. 73, 465-471.
- Quiroz-Guzman, E., Balcazar, J.L., Vazquez-Juarez, R., Cruz-Villacorta, A.A., Martinez-Diaz, S.F., 2013. Proliferation, colonization, and detrimental effects of *Vibrio parahaemolyticus* and *Vibrio harveyi* during brine shrimp hatching. *Aquaculture*. 406, 85-90.
- R Core Team, 2015. R: A language and environment for statistical computing. Available from: <http://www.R-project.org/>.
- Raab, O., 1900. Über die wirkung fluoreszierender stoffe auf infusorien. *Zeitschrift fuer Biologie*. 39, 524-546.
- Ragas, X., He, X., Agut, M., Roxo-Rosa, M., Gonsalves, A.R., Serra, A.C., Nonell, S., 2013. Singlet oxygen in antimicrobial photodynamic therapy: photosensitizer dependent production and decay in *E. coli*. *Molecules*. 18, 2712-2725.
- Ramanan, R., Kim, B.-H., Cho, D.-H., Oh, H.-M., Kim, H.-S., 2016. Algae–bacteria interactions: Evolution, ecology and emerging applications. *Biotechnology Advances*. 34, 14-29.
- Reinke, D.C., 1984. Ultrastructure of *Chaetoceros muelleri* (Bacillariophyceae): Auxospore, resting spore and vegetative cell morphology. *Journal of Phycology*. 20, 153-155.
- Reitan, K.I., Rainuzzo, J.R., Olsen, Y., 1994. Effect of nutrient limitation on fatty-acid and lipid-content of marine microalgae. *Journal of Phycology*. 30, 972-979.
- Renaud, S.M., Thinh, L.V., Parry, D.L., 1999. The gross chemical composition and fatty acid composition of 18 species of tropical Australian microalgae for possible use in mariculture. *Aquaculture*. 170, 147-159.
- Renaud, S.M., Thinh, L.V., Lambrinidis, G., Parry, D.L., 2002. Effect of temperature on growth, chemical composition and fatty acid composition of tropical Australian microalgae grown in batch cultures. *Aquaculture*. 211, 195-214.
- Rengifo-Herrera, J.A., Sanabria, J., Machuca, F., Dierolf, C.F., Pulgarin, C., Orellana, G., 2005. A comparison of solar photocatalytic inactivation of waterborne *E. coli*

- using tris (2,2'-bipyridine)ruthenium(ii), rose bengal, and TiO₂. *Journal of Solar Energy Engineering*. 129, 135-140.
- Rhodes, L.D., Johnson, R.B., Myers, M.S., 2016. Effects of alternative plant-based feeds on hepatic and gastrointestinal histology and the gastrointestinal microbiome of sablefish (*Anoplopoma fimbria*). *Aquaculture*. 464, 683-691.
- Ribeiro, F.A.L.T., Jones, D.A., 1998. The potential of dried, low-hatch, decapsulated *Artemia* cysts for feeding prawn post-larvae. *Aquaculture International*. 6, 421-440.
- Ritz, C., Streibig, J.C., 2005. Bioassay analysis using R. *Journal of Statistical Software*. 12, 1-22.
- Ritz, C., Cedergreen, N., Jensen, J.E., Streibig, J.C., 2006. Relative potency in nonsimilar dose-response curves. *Weed Science*. 54, 407-412.
- Ritz, C., Baty, F., Streibig, J.C., Gerhard, D., 2015. Dose-response analysis using R. *PLoS ONE*. 10, e0146021.
- Ritz, C., Streibig, J.C., 2016. drc: Analysis of Dose-Response Curves. R package.
- Rivers, T.M., 1937. Viruses and Koch's postulates. *Journal of Bacteriology*. 33, 1-12.
- Robbins, H.M., Van Stappen, G., Sorgeloos, P., Sung, Y.Y., MacRae, T.H., Bossier, P., 2010. Diapause termination and development of encysted *Artemia* embryos: roles for nitric oxide and hydrogen peroxide. *The Journal of Experimental Biology*. 213, 1464-1470.
- Robertson, C., 2006. Australian prawn farming manual: Health management for profit. in: Robertson, C. (Ed.). *The State of Queensland, Department of Primary Industries and Fisheries, Brisbane*.
- Robinson, J.R., 1983. Photodynamic insecticides: A review of studies on photosensitizing dyes as insect control agents, their practical application, hazards, and residues, in: Gunther, F.A. (Ed.), *Residue Reviews: Residues of Pesticides and Other Contaminants in the Total Environment*. Springer New York, New York, NY, pp. 69-100.
- Rodgers, M.A.J., Snowden, P.T., 1982. Lifetime of oxygen O₂(¹Δ_g) in liquid water as determined by time-resolved infrared luminescence measurements. *Journal of the American Chemical Society*. 104, 5541-5543.
- Romalde, J.L., 2002. *Photobacterium damsela* subsp. *piscicida*: An integrated view of a bacterial fish pathogen. *International Microbiology*. 5, 3-9.
- Roots, R., Okada, S., 1975. Estimation of life times and diffusion distances of radicals involved in x-ray-induced DNA strand breaks of killing of mammalian cells. *Radiation Research*. 64, 306-320.
- Rotllant, J., Pavlidis, M., Kentouri, M., Adad, M.E., Tort, L., 1997. Non-specific immune responses in the red porgy *Pagrus pagrus* after crowding stress. *Aquaculture*. 156, 279-290.

- Rotomskis, R., Streckyte, G., Bagdonas, S., 1997. Phototransformations of sensitizers .2. Photoproducts formed in aqueous solutions of porphyrins. *Journal of Photochemistry and Photobiology B: Biology*. 39, 172-175.
- Rowley, A.F., Pope, E.C., 2012. Vaccines and crustacean aquaculture—A mechanistic exploration. *Aquaculture*. 334–337, 1-11.
- Royal Society of Chemistry, 2007. Sustainable water summary report: chemical science priorities. Burlington House, London, pp. 1-26.
- RStudio Team, 2015. RStudio: integrated development for R, Boston, MA.
- Rupert G, M.J., 1981. Simultaneous statistical inference. Springer, 978-1-4613-8124-2.
- Ryter, S.W., Tyrrell, R.M., 1998. Singlet molecular oxygen (1O_2): a possible effector of eukaryotic gene expression. *Free Radical Biology and Medicine*. 24, 1520-1534.
- Sabbahi, S., Ben Ayed, L., Boudabbous, A., 2013. Cationic, anionic and neutral dyes: effects of photosensitizing properties and experimental conditions on the photodynamic inactivation of pathogenic bacteria. *Journal of Water and Health*. 11, 590-599.
- Sahul Hameed, A.S., Balasubramanian, G., 2000. Antibiotic resistance in bacteria isolated from *Artemia* nauplii and efficacy of formaldehyde to control bacterial load. *Aquaculture*. 183, 195-205.
- Salvesen, I., Skjermo, J., Vadstein, O., 1999. Growth of turbot (*Scophthalmus maximus* L.) during first feeding in relation to the proportion of r/K-strategists in the bacterial community of the rearing water. *Aquaculture*. 175, 337-350.
- Salvesen, I., Reitan, K.I., Skjermo, J., Oie, G., 2000. Microbial environments in marine larviculture: Impacts of algal growth rates on the bacterial load in six microalgae. *Aquaculture International*. 8, 275-287.
- Saoud, I.P., Ghanawi, J., Thompson, K.R., Webster, C.D., 2013. A review of the culture and diseases of redclaw crayfish *Cherax quadricarinatus* (Von Martens 1868). *Journal of the World Aquaculture Society*. 44, 1-29.
- Saravanan, K., Nilavan, S.E., Sudhagar, S.A., Naveenchandru, V., 2013. Diseases of mariculture finfish species: A review. *Israeli Journal of Aquaculture-Bamidgeh*. 65, 1-14.
- Sarker, M.S.A., Satoh, S., Kamata, K., Haga, Y., Yamamoto, Y., 2012. Partial replacement of fish meal with plant protein sources using organic acids to practical diets for juvenile yellowtail, *Seriola quinqueradiata*. *Aquaculture Nutrition*. 18, 81-89.
- Saulnier, D., Haffner, P., Goarant, C., Levy, P., Ansquer, D., 2000. Experimental infection models for shrimp vibriosis studies: a review. *Aquaculture*. 191, 133-144.
- Sawabe, T., Kita-Tsukamoto, K., Thompson, F.L., 2007. Inferring the evolutionary history of vibrios by means of multilocus sequence analysis. *Journal of Bacteriology*. 189, 7932-7936.
- Schafer, M., 1998. High sensitivity of *Deinococcus radiodurans* to photodynamically produced singlet oxygen. *International Journal of Radiation Biology*. 74, 249-253.

- Scholz, M.J., Weiss, T.L., Jinkerson, R.E., Jing, J., Roth, R., Goodenough, U., Posewitz, M.C., Gerken, H.G., 2014. Ultrastructure and composition of the *Nannochloropsis gaditana* cell wall. *Eukaryotic Cell*. 13, 1450-1464.
- Schrader, K.K., Bommer, J.C., Jori, G., 2010. In vitro evaluation of the antimicrobial agent AquaFrin as a bactericide and selective algicide for use in channel catfish aquaculture. *North American Journal of Aquaculture*. 72, 304-308.
- Sellera, F.P., Sabino, C.P., Ribeiro, M.S., Fernandes, L.T., Pogliani, F.C., Teixeira, C.R., Dutra, G.H., Nascimento, C.L., 2014. Photodynamic therapy for pododermatitis in penguins. *Zoo Biology*. 33, 353-356.
- Sensen, C.W., Heimann, K., Melkonian, M., 1993. The production of clonal and axenic cultures of microalgae using fluorescence-activated cell sorting. *European Journal of Phycology*. 28, 93-97.
- Seoane, M., Rioboo, C., Cid, n., 2014. Toxicity of three antibiotics used in aquaculture on the marine microalgae *Tetraselmis suecica* (Kylin) Butch. *Frontiers in Marine Science*.
- Sharma, P., Jha, A.B., Dubey, R.S., Pessarakli, M., 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *Journal of Botany*. 2012, 26.
- Sharma, S.K., Chiang, L.Y., Hamblin, M.R., 2011. Photodynamic therapy with fullerenes in vivo: reality or a dream? *Nanomedicine*. 6, 1813-1825.
- Shivu, M.M., Rajeeva, B.C., Girisha, S.K., Karunasagar, I., Krohne, G., Karunasagar, I., 2007. Molecular characterization of *Vibrio harveyi* bacteriophages isolated from aquaculture environments along the coast of India. *Environmental Microbiology*. 9, 322-331.
- Shrestha, A., Kishen, A., 2012. Polycationic chitosan-conjugated photosensitizer for antibacterial photodynamic therapy. *Photochemistry and photobiology*. 88, 577-583.
- Shrimp News International, 2014. About Shrimp Farming. <http://www.shrimpnews.com/FreeReportsFolder/GeneralInformationFolder/About.html>.
- Silva, J.N., Filipe, P., Morliere, P., Maziere, J.C., Freitas, J.P., Gomes, M.M., Santus, R., 2008. Photodynamic therapy: Dermatology and ophthalmology as main fields of current applications in clinic. *Biomedical Material and Engineering*. 18, 319-327.
- Sing, S.F., Isdepsky, A., Borowitzka, M.A., Moheimani, N.R., 2013. Production of biofuels from microalgae. *Mitigation and Adaptation Strategies for Global Change*. 18, 47-72.
- Skjermo, J., Vadstein, O., 1993. The effect of microalgae on skin and gut bacterial-flora of halibut larvae. *Fish Farming Technology*, 61-67.
- Skovsen, E., Snyder, J.W., Lambert, J.D.C., Ogilby, P.R., 2005. Lifetime and diffusion of singlet oxygen in a cell. *Journal of Physical Chemistry B*. 109, 8570-8573.

- Smith, G., Salmon, M., Kenway, M., Hall, M., 2009. Description of the larval morphology of captive reared *Panulirus ornatus* spiny lobsters, benchmarked against wild-caught specimens. *Aquaculture*. 295, 76-88.
- Snyder, J.W., Skovsen, E., Lambert, J.D.C., Poulsen, L., Ogilby, P.R., 2006. Optical detection of singlet oxygen from single cells. *Physical Chemistry Chemical Physics*. 8, 4280-4293.
- Sorgeloos, P., Bossuyt, E., Laviña, E., Baeza-Mesa, M., Persoone, G., 1977. Decapsulation of *Artemia* cysts: A simple technique for the improvement of the use of brine shrimp in aquaculture. *Aquaculture*. 12, 311-315.
- Sorgeloos, P., 1980. The use of the brine shrimp *Artemia* in aquaculture, in: Persoone, G., Sorgeloos, P., Roels, O., Jaspers, E. (Eds.), *The brine shrimp Artemia*. Universa Press, Wetteren, Belgium, pp. 3–24.
- Sorgeloos, P., Coutteau, P., Dhert, P., Merchie, G., Lavens, P., 1998. Use of brine shrimp, *Artemia* spp., in larval crustacean nutrition: A review. *Reviews in Fisheries Science*. 6, 55-68.
- Sorgeloos, P., Dhert, P., Candreva, P., 2001. Use of the brine shrimp, *Artemia* spp., in marine fish larviculture. *Aquaculture*. 200, 147-159.
- Sperandio, F.F., Huang, Y.-Y., Hamblin, M.R., 2013. Antimicrobial photodynamic therapy to kill Gram-negative bacteria. *Recent Patents on Anti-Infective Drug Discovery*. 8, 108-120.
- Stael, M., Sanggontanagit, T., Van Ballaer, E., Puwapanich, N., Tunsutapanicha, A., Lavens, P., 1995. Decapsulated cysts and *Artemia* flakes as alternative food sources for the culture of *Penaeus monodon* postlarvae. in: Lavens, P., Jaspers, E., I., R. (Eds.), *Larvi'95-Fish and Shellfish Larviculture Symposium*. European Aquaculture Society Special Publication, Gent, Belgium., pp. 342–345.
- Stojiljkovic, I., Evavold, B.D., Kumar, V., 2001. Antimicrobial properties of porphyrins. *Expert Opinion on Investigational Drugs*. 10, 309-320.
- Suantika, G., Dhert, P., Rombaut, G., Vandenberghe, J., De Wolf, T., Sorgeloos, P., 2001. The use of ozone in a high density recirculation system for rotifers. *Aquaculture*. 201, 35-49.
- Suga, K., Tanaka, Y., Sakakura, Y., Hagiwara, A., 2011. Axenic culture of *Brachionus plicatilis* using antibiotics. *Hydrobiologia*. 662, 113-119.
- Sung, H.H., Hsu, S.F., Chen, C.K., Ting, Y.Y., Chao, W.L., 2001. Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. *Aquaculture*. 192, 101-110.
- Sung, Y.Y., MacRae, T.H., Sorgeloos, P., Bossier, P., 2011. Stress response for disease control in aquaculture. *Reviews in Aquaculture*. 3, 120-137.
- Suzuki, N., Mizumoto, I., Itami, T., Takahashi, Y., Tanaka, R., Hatate, H., Nomoto, T., Kozawa, A., 2000. Virucidal effect of singlet oxygen on penaeid white spot syndrome virus. *Fisheries Science*. 66, 166-168.

- Sykora, J.L., Keleti, G., Roche, R., Volk, D.R., Kay, G.P., Burgess, R.A., Shapiro, M.A., Lippy, E.C., 1980. Endotoxins, algae and *Limulus amoebocyte* lysate test in drinking water. *Water Research*. 14, 829-839.
- Szeimies, R.-M., Dräger, J., Abels, C., Landthaler, M., 2001. History of photodynamic therapy in dermatology. *Comprehensive Series in Photosciences*. 2, 3-15.
- Szeimies, R.M., Lischner, S., Philipp-Dormston, W., Walker, T., Hiepe-Wegener, D., Feise, K., Podda, M., Prager, W., Kohl, E., Karrer, S., 2013. Photodynamic therapy for skin rejuvenation: treatment options - results of a consensus conference of an expert group for aesthetic photodynamic therapy. *Journal of the German Society of Dermatology*. 11, 632-636.
- Tacon, A.G.J., Metian, M., Turchini, G.M., De Silva, S.S., 2009. Responsible aquaculture and trophic level implications to global fish supply. *Reviews in Fisheries Science*. 18, 94-105.
- Taub, F.A., 2012. Cosmetic clinical indications for photodynamic therapy. *Journal of Cosmetic Dermatology*. 25, 218-224.
- Tavares, A., Carvalho, C.M.B., Faustino, M.A., Neves, M., Tome, J.P.C., Tome, A.C., Cavaleiro, J.A.S., Cunha, A., Gomes, N.C.M., Alves, E., Almeida, A., 2010. Antimicrobial photodynamic therapy: Study of bacterial recovery viability and potential development of resistance after treatment. *Marine Drugs*. 8, 91-105.
- Tavares, A., Dias, S.R.S., Carvalho, C.M.B., Faustino, M.A.F., Tome, J.P.C., Neves, M., Tome, A.C., Cavaleiro, J.A.S., Cunha, A., Gomes, N.C.M., Alves, E., Almeida, A., 2011. Mechanisms of photodynamic inactivation of a Gram-negative recombinant bioluminescent bacterium by cationic porphyrins. *Photochemical and Photobiological Sciences*. 10, 1659-1669.
- Tazawa, E., Iwanami, Y., 1974. Hatching of brine-shrimp eggs (*Artemia salina*) soaked in organic solvents. *Zoological Magazine*.
- Teplitski, M., Rajamani, S., 2011. Signal and nutrient exchange in the interactions between soil algae and bacteria, in: Witzany, G. (Ed.), *Biocommunication in Soil Microorganisms*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 413-426.
- Thompson, F.L., Iida, T., Swings, J., 2004. Biodiversity of vibrios. *Microbiology and Molecular Biology Reviews*. 68, 403-+.
- Thompson, F.L., Gevers, D., Thompson, C.C., Dawyndt, P., Naser, S., Hoste, B., Munn, C.B., Swings, J., 2005. Phylogeny and molecular identification of vibrios on the basis of multilocus sequence analysis. *Applied and Environmental Microbiology*. 71, 5107-5115.
- Thompson, P.A., Guo, M.X., Harrison, P.J., 1992. Effects of variation in temperature .1. On the biochemical-composition of 8 species of marine-phytoplankton. *Journal of Phycology*. 28, 481-488.
- Thornton, D.C.O., 2009. Effect of low pH on carbohydrate production by a marine planktonic diatom (*Chaetoceros muelleri*). *Research Letters in Ecology*. doi:10.1155/2009/105901, 1-4.

- Tolomei, A., Burke, C., Crear, B., Carson, J., 2004. Bacterial decontamination of on-grown *Artemia*. *Aquaculture*. 232, 357-371.
- Toranzo, A.E., Barreiro, S., Casal, J.F., Figueras, A., Magarinos, B., Barja, J.L., 1991. Pasteurellosis in cultured gilthead seabream (*Sparus-aurata*) - 1st report in Spain. *Aquaculture*. 99, 1-15.
- Toranzo, A.E., Magarinos, B., Romalde, J.L., 2005. A review of the main bacterial fish diseases in mariculture systems. *Aquaculture*. 246, 37-61.
- Tubiash, H.S., Chanley, P.E., Leifson, E., 1965. Bacillary necrosis a disease of larval and juvenile bivalve mollusks .1. etiology and epizootiology. *Journal of Bacteriology*. 90, 1036-&.
- Tunsutapanich, A., 1979. An improved technique for decapsulation and preservation of *Artemia* cysts (Brine shrimp eggs) developed at the Chachoengsao Fisheries Station in Thailand. in: Division, N.F.P.R.a.T.C.I.F. (Ed.). Ministry of Agriculture and Cooperatives.
- Tye, M., Rider, D., Duffy, E.A., Seubert, A., Lothert, B., Schimmenti, L.A., 2014. Nonhatching decapsulated *Artemia* cysts as a replacement to *Artemia* nauplii in juvenile and adult zebrafish culture. *Zebrafish*. 12, 457-461.
- Umamaheswari, J., Shanthakumar, S., 2016. Efficacy of microalgae for industrial wastewater treatment: a review on operating conditions, treatment efficiency and biomass productivity. *Reviews in Environmental Science and Biotechnology*. 15, 265-284.
- Unnithan, V.V., Unc, A., Smith, G.B., 2014. Mini-review: A priori considerations for bacteria-algae interactions in algal biofuel systems receiving municipal wastewaters. *Algal Research*. 4, 35-40.
- Urbanczyk, H., Ogura, Y., Hayashi, T., 2013. Taxonomic revision of Harveyi clade bacteria (family Vibrionaceae) based on analysis of whole genome sequences. *International Journal of Systematic and Evolutionary Microbiology*. 63, 2742-2751.
- Vadstein, O., 1997. The use of immunostimulation in marine larviculture: Possibilities and challenges. *Aquaculture*. 155, 401-417.
- Valkov, A., Nakonechny, F., Nisnevitch, M., 2014. Polymer-Immobilized Photosensitizers for Continuous Eradication of Bacteria. *International Journal of Molecular Sciences*. 15, 14984-14996.
- Van Bleijswijk, J.D.L., Veldhuis, M.J.W., 1995. In situ gross growth rates of *Emiliania huxleyi* in enclosures with different phosphate loadings revealed by diel changes in DNA content. *Marine Ecology Progress Series*. 121, 271-277.
- Van Stappen, G., 1996. Introduction, biology and ecology of *Artemia*, in: Lavens, P., Sorgeloos, P. (Eds.), *Manual on the production and use of live food for aquaculture*. FAO, pp. 79-106.
- Van Stappen, G., Lavens, P., Sorgeloos, P., 1998. Effects of hydrogen peroxide treatment in *Artemia* cysts of different geographical origin. *Fundamental and Applied Limnology*. 52, 281-296.

- Van Stappen, G., 2002. Zoogeography, in: Abatzopoulos, T., Beardmore, J., Clegg, J., Sorgeloos, P. (Eds.), *Artemia: Basic and Applied Biology*. Springer, pp. 171-224.
- Vandekerckhove, B., Parmentier, L., Van Stappen, G., Grenier, S., Febvay, G., Rey, M., De Clercq, P., 2009. *Artemia* cysts as an alternative food for the predatory bug *Macrolophus pygmaeus*. *Journal of Applied Entomology*. 133, 133-142.
- Vandenbergh, J., Verdonck, L., Robles-Arozarena, R., Rivera, G., Bolland, A., Balladares, M., Gomez-Gil, B., Calderon, J., Sorgeloos, P., Swings, J., 1999. Vibrios associated with *Litopenaeus vannamei* larvae, postlarvae, broodstock, and hatchery probionts. *Applied and Environmental Microbiology*. 65, 2592-2597.
- Vanhaecke, P., Lavens, P., Sorgeloos, P., 1983. International study on *Artemia*: XVII. Energy consumption in cysts and early larval stages of various geographical strains of *Artemia*. *Annales de la Société Royale Zoologique de Belgique*. 113, 155-164.
- Vanmaele, S., Defoirdt, T., Cleenwerck, I., De Vos, P., Bossier, P., 2015. Characterization of the virulence of Harveyi clade vibrios isolated from a shrimp hatchery in vitro and in vivo, in a brine shrimp (*Artemia franciscana*) model system. *Aquaculture*. 435, 28-32.
- Vargasalbores, F., Guzman, M.A., Ochoa, J.L., 1993. A lipopolysaccharide-binding agglutinin isolated from brown shrimp (*Penaeus-californiensis holmes*) hemolymph. *Comparative Biochemistry and Physiology B: Biochemistry and Molecular Biology*. 104, 407-413.
- Vatansever, F., De Melo, W.C.M.A., Avci, P., Vecchio, D., Sadasivam, M., Gupta, A., Chandran, R., Karimi, M., Parizotto, N.A., Yin, R., Tegos, G.P., Hamblin, M.R., 2013. Antimicrobial strategies centered around reactive oxygen species - bactericidal antibiotics, photodynamic therapy, and beyond. *FEMS Microbiology Reviews*. 37, 955-989.
- Veldhuis, M.J.W., Kraay, G.W., 2000. Application of flow cytometry in marine phytoplankton research: Current applications and future perspectives. *Scientia Marina*. 64, 121-134.
- Velmurugan, S., Palanikumar, P., Velayuthani, P., Donio, M.B.S., Babu, M.M., Lelin, C., Sudhakar, S., Citarasu, T., 2015. Bacterial white patch disease caused by *Bacillus cereus*, a new emerging disease in semi-intensive culture of *Litopenaeus vannamei*. *Aquaculture*. 444, 49-54.
- Verdonck, L., Grisez, L., Sweetman, E., Minkoff, G., Sorgeloos, P., Ollevier, F., Swings, J., 1997. Vibrios associated with routine productions of *Brachionus plicatilis*. *Aquaculture*. 149, 203-214.
- von Alvensleben, N., Stookey, K., Magnusson, M., Heimann, K., 2013. Salinity tolerance of *Picochlorum atomus* and the use of salinity for contamination control by the freshwater cyanobacterium *Pseudanabaena limnetica*. *Plos One*. 8, 12.
- Von Tappeiner, H., 1904. Zur kenntnis der lichtwirkenden (fluoreszierenden) stoffe. *Deutsche Medizinische Wochenschau*. 30, 579-580.

- Von Tappeiner, H., 1909. Die photodynamische erscheinung (sensibilisierung durch fluoreszierende stoffe). *Ergebnisse der Physiologie*. 8, 698-741.
- Wainwright, M., 2002. Pathogen inactivation in blood products. *Current Medicinal Chemistry*. 9, 127-143.
- Wainwright, M., 2003. Local treatment of viral disease using photodynamic therapy. *International Journal of Antimicrobial Agents*. 21, 510-520.
- Wainwright, M., Crossley, K.B., 2004. Photosensitising agents—circumventing resistance and breaking down biofilms: a review. *International Biodeterioration and Biodegradation*. 53, 119-126.
- Waksman, R., McEwan, P.E., Moore, T.I., Pakala, R., Kolodgie, F.D., Hellinga, D.G., Seabron, R.C., Rychnovsky, S.J., Vasek, J., Scott, R.W., Virmani, R., 2008. Photopoint photodynamic therapy promotes stabilization of atherosclerotic plaques and inhibits plaque progression. *Journal of the American College of Cardiology*. 52, 1024-1032.
- Wan, M.T., Lin, J.Y., 2014. Current evidence and applications of photodynamic therapy in dermatology. *Clinical, Cosmetic and Investigational Dermatology*. 7, 145-163.
- Wang, L.P., Chen, Y.W., Huang, H., Huang, Z.B., Chen, H., Shao, Z.Z., 2015. Isolation and identification of *Vibrio campbellii* as a bacterial pathogen for luminous vibriosis of *Litopenaeus vannamei*. *Aquaculture Research*. 46, 395-404.
- Wang, X., Zhang, Y., Qin, G., Luo, W., Lin, Q., 2016. A novel pathogenic bacteria (*Vibrio fortis*) causing enteritis in cultured seahorses, *Hippocampus erectus* Perry, 1810. *Journal of Fish Diseases*. 39, 765-769.
- Wardlaw, J.L., Sullivan, T.J., Lux, C.N., Austin, F.W., 2012. Photodynamic therapy against common bacteria causing wound and skin infections. *The Veterinary Journal*. 192, 374-377.
- Watanabe, K., Takihana, N., Aoyagi, H., Hanada, S., Watanabe, Y., Ohmura, N., Saiki, H., Tanaka, H., 2005. Symbiotic association in *Chlorella* culture. *FEMS Microbiology Ecology*. 51, 187-196.
- Watts, R.J., Kong, S., Orr, M.P., Miller, G.C., Henry, B.E., 1995. Photocatalytic inactivation of coliform bacteria and viruses in secondary wastewater effluent. *Water Research*. 29, 95-100.
- Whyte, J.N.C., 1987. Biochemical-composition and energy content of 6 species of phytoplankton used in mariculture of bivalves. *Aquaculture*. 60, 231-241.
- Wiedeman, V.E., Walne, P.L., Trainor, F.R., 1964. A new technique for obtaining axenic cultures of algae. *Canadian Journal of Botany*. 42, 958-959.
- Wietz, M., Hall, M.R., Hoj, L., 2009. Effects of seawater ozonation on biofilm development in aquaculture tanks. *Systematic and Applied Microbiology*. 32, 266-277.
- Wikene, K.O., Bruzell, E., Tonnesen, H.H., 2015. Improved antibacterial phototoxicity of a neutral porphyrin in natural deep eutectic solvents. *Journal of Photochemistry and Photobiology B: Biology*. 148, 188-196.

- Wilkie, A.C., Edmundson, S.J., Duncan, J.G., 2011. Indigenous algae for local bioresource production: Phycoprospecting. *Energy for Sustainable Development*. 15, 365-371.
- Withers, P.C., 1992. *Comparative animal physiology*. Saunders College Publishing, Sydney, NSW, Australia, 0030128471.
- Wohllebe, S., Richter, P., Haeder, D.P., 2012. Chlorophyllin for the control of *Ichthyophthirius multifiliis* (Fouquet). *Parasitology Research*. 111, 729-733.
- Woodburn, K.W., Fan, Q., Kessel, D., Wright, M., Mody, T.D., Hemmi, G., Magda, D., Sessler, J.L., Dow, W.C., Miller, R.A., Young, S.W., 1996. Phototherapy of cancer and atheromatous plaque with texaphyrins. *Journal of Clinical Laser Medicine and Surgery* 14, 343-348.
- Yamamoto, M., Nozaki, H., Miyazawa, Y., Koide, T., Kawano, S., 2003. Relationship between presence of a mother cell wall and speciation in the unicellular microalga *Nannochloris* (Chlorophyta). *Journal of Phycology*. 39, 172-184.
- Yim, J.H., Lee, H.K., 2004. Axenic culture of *Gyrodinium impudicum* strain KG03, a marine red-tide microalga that produces exopolysaccharide. *Journal of microbiology*. 42, 305-314.
- Yoon, I., Li, J.Z., Shim, Y.K., 2013. Advance in photosensitizers and light delivery for photodynamic therapy. *Clinical endoscopy*. 46, 7-23.
- Youn, J.-Y., Hur, S.-B., 2007. Antibiotics and their optimum concentration for axenic culture of marine microalgae. *Algae*. 22, 229-234.
- Zanetti, S., Spanu, T., Deriu, A., Romano, L., Sechi, L.A., Fadda, G., 2001. In vitro susceptibility of *Vibrio* spp. isolated from the environment. *International Journal of Antimicrobial Agents*. 17, 407-409.
- Zeina, B., Greenman, J., Purcell, W.M., Das, B., 2001. Killing of cutaneous microbial species by photodynamic therapy. *British Journal of Dermatology*. 144, 274-278.
- Zhang, L.T., Li, L., Liu, J.G., 2014a. Comparison of the photosynthetic characteristics of two *Isochrysis galbana* strains under high light. *Botanica Marina*. 57, 477-481.
- Zhang, W.W., Li, C.H., 2016. Exploiting quorum sensing interfering strategies in gram-negative bacteria for the enhancement of environmental applications. *Frontiers in Microbiology*. 6, 15.
- Zhang, X.J., Bai, X.S., Yan, B.L., Bi, K.R., Qin, L., 2014b. *Vibrio harveyi* as a causative agent of mass mortalities of megalopa in the seed production of swimming crab *Portunus trituberculatus*. *Aquaculture International*. 22, 661-672.
- Zheng, C.Q., Jeswin, J., Shen, K.L., Lablche, M., Wang, K.J., Liu, H.P., 2015. Detrimental effect of CO₂-driven seawater acidification on a crustacean brine shrimp, *Artemia sinica*. *Fish and Shellfish Immunology*. 43, 181-190.

Appendices

A2 APPENDIX CHAPTER 2

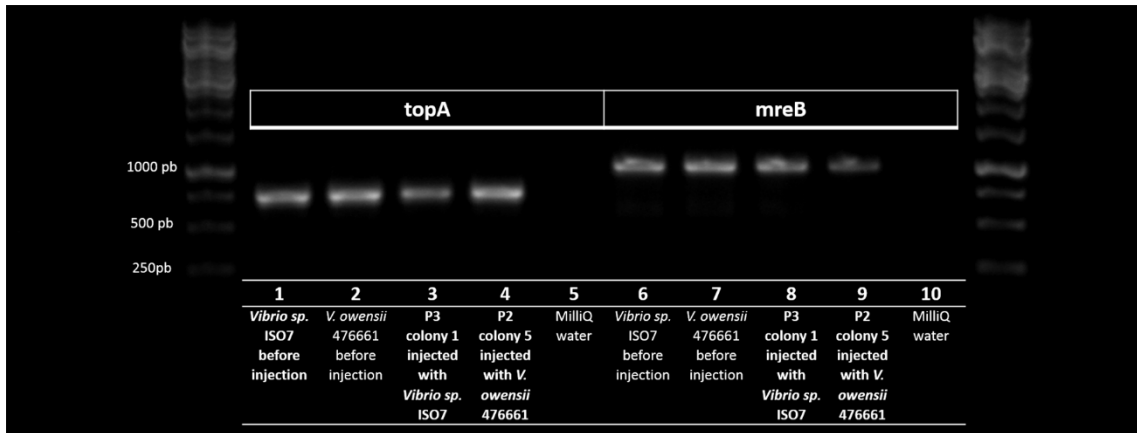


Figure A2.1: Separation of PCR product in agarose gel after *topA* and *mreB* genes amplification from selected sample in challenge experiment 1. DNA ladder (1kbp) in the edge of the gel.

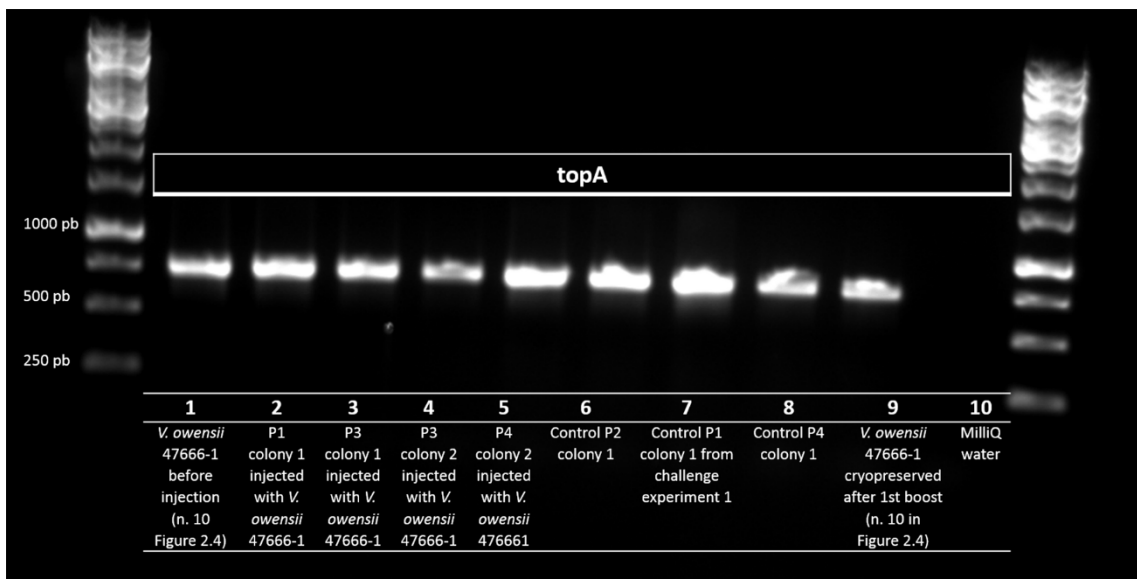


Figure A2.2: Product separation in agarose gel after *topA* gene amplification from selected sample in challenge experiment 2. Selected colonies of plated haemolymph from prawns (P1 to P4) in Figure 2.6 and Figure 2.7. The DNA was extracted and gene amplified as described in 2.2.7. DNA ladder (1k bp) at the edge of the gel.

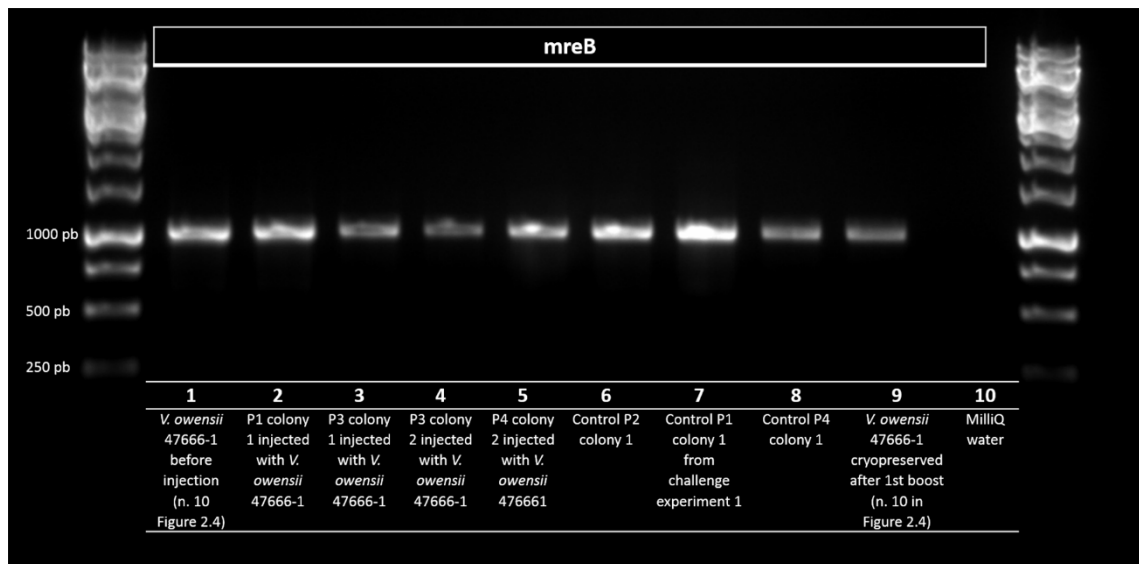


Figure A2.3: Product separation in agarose gel after *mreB* gene amplification from selected sample in challenge experiment 2. Selected colonies of plated haemolymph from prawns (P1 to P4) in Figure 2.6 and Figure 2.7. The DNA was extracted and gene amplified as described in 2.2.7. DNA ladder (1k bp) at the edge of the gel.

A3 APPENDIX CHAPTER 3

A3.1 PHOTBLEACHING EXPERIMENT

Table A3.1: Shapiro-Wilk normality test of each treatment in the photobleaching experiment (ABS_{425}) over 24 h of irradiation time using TMPyP porphyrin.

Treatment	W	p-value
20 μ M	0.86	4.53×10^{-02}
10 μ M	0.83	2.27×10^{-02}
5 μ M	0.85	3.87×10^{-02}
1 μ M	0.84	2.87×10^{-02}
Dark control	0.69	7.41×10^{-04}

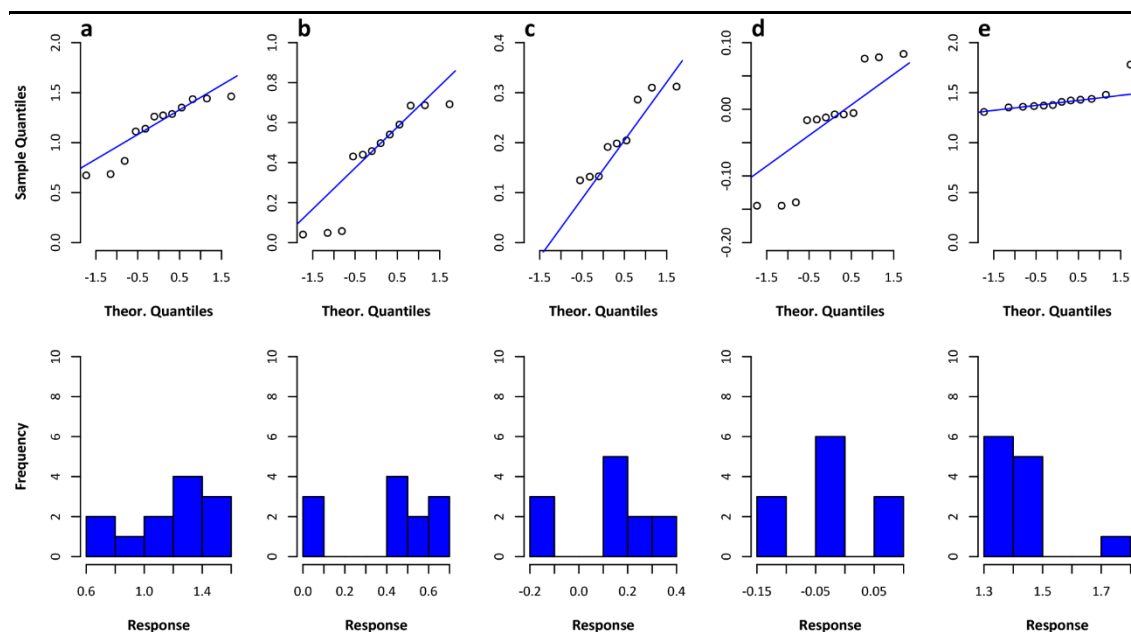


Figure A3.1: Graphic representation of normality distribution of each treatment in the photobleaching experiment (ABS_{425}) over 24 h of irradiation time using TMPyP. Top = Q-Q plots of sample quintiles against theoretical quantiles, Bottom = Histogram using frequency against response. Treatment: a = 20 μ M, b = 10 μ M, c = 5 μ M, d = 1 μ M, e = Dark control (20 μ M in the dark).

Table A3.2: Levene's Test results in photobleaching experiment (ABS_{425}) using TMPyP. Independent variables: Treatment (over 24 h) and time for each porphyrin concentration.

Treatments	Variable	Df	F-value	Pr(>F)
	Treatment	4	4.86	1.99×10^{-03}
20 μ M	Time	3	3.83	5.72×10^{-02}
10 μ M	Time	3	2.91	0.10
5 μ M	Time	3	4.30	4.39×10^{-02}
1 μ M	Time	3	1.77	0.23
Dark control (20 μ M)	Time	3	5.27	2.68×10^{-02}

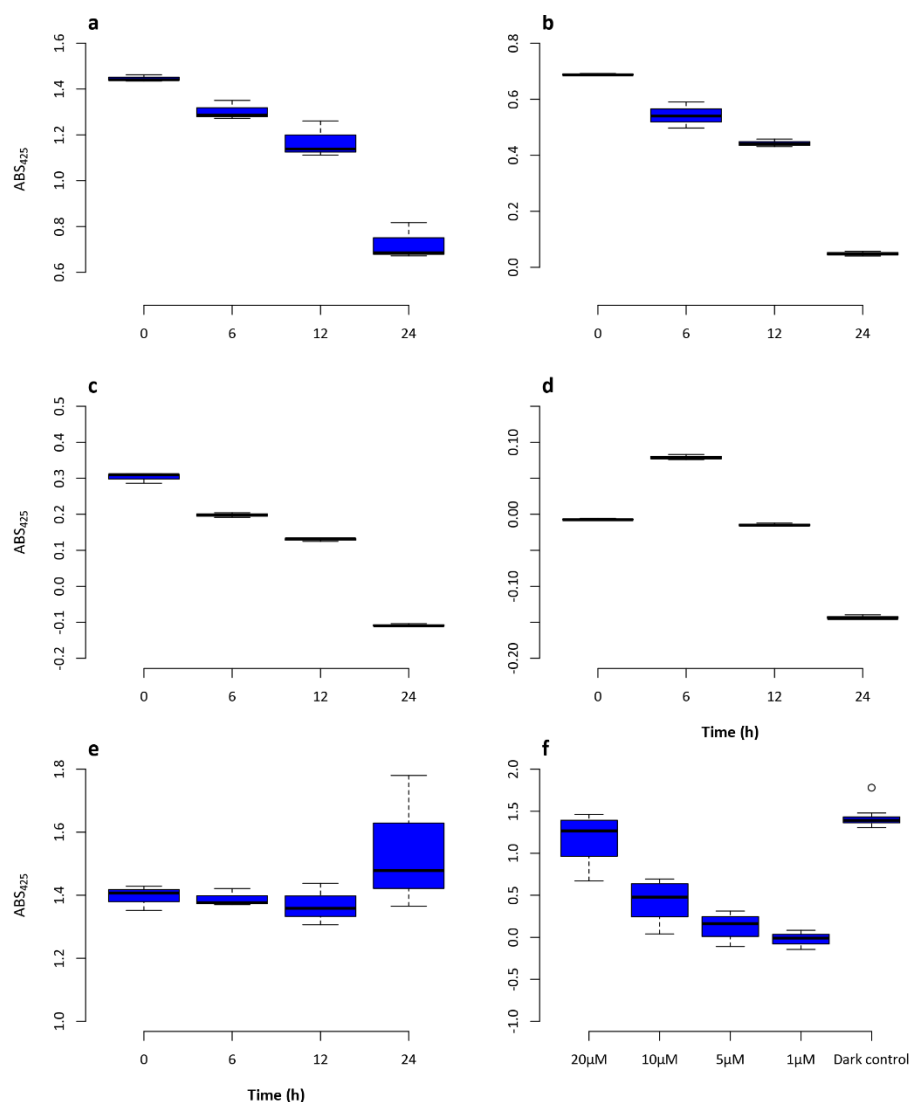


Figure A3.2: Boxplot showing homogeneity of variance in photobleaching experiment (ABS_{425}) using TMPyP. Independent variables: time (a-f) and treatments (g). Figure: a = 20 μM , b = 10 μM , c = 5 μM , d = 1 μM , e = Light control (0 μM), f = Dark control (20 μM in the dark), g = all treatment over 24 h.

Table A3.3: Shapiro-Wilk normality test of each treatment in the photobleaching experiment (ABS_{414}) over 24 h of irradiation time using TPPS₄ porphyrin.

Treatment	W	p-value
20 μM	0.95	0.57
10 μM	0.90	0.17
5 μM	0.91	0.23
1 μM	0.87	0.07
Dark control	0.94	0.53

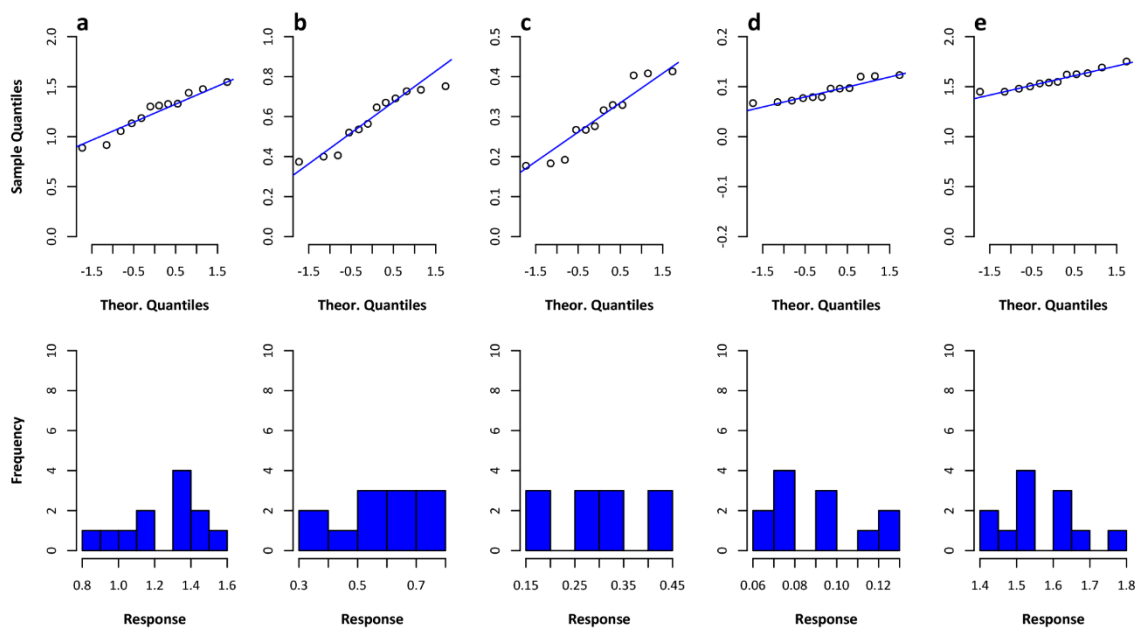


Figure A3.3: Graphic representation of normality distribution of each treatment in the photobleaching experiment (ABS_{414}) over 24 h of irradiation time using TPPS₄. Top = Q-Q plots of sample quantiles against theoretical quantiles, Bottom = Histogram using frequency against response. Treatment: a = 20 μ M, b = 10 μ M, c = 5 μ M, d = 1 μ M, e = Dark control (20 μ M in the dark).

Table A3.4: Levene's Test results in photobleaching experiment (ABS_{414}) using TPPS₄. Independent variables: Treatment (over 24 h) and time for each porphyrin concentration.

Treatments	Variable	Df	F-value	Pr(>F)
	Treatment	4	9.74	4.96×10^{-06}
20 μ M	Time	3	3.15	0.09
10 μ M	Time	3	0.30	0.82
5 μ M	Time	3	0.49	0.70
1 μ M	Time	3	1.64	0.26
Dark control (20 μ M)	Time	3	0.65	0.60

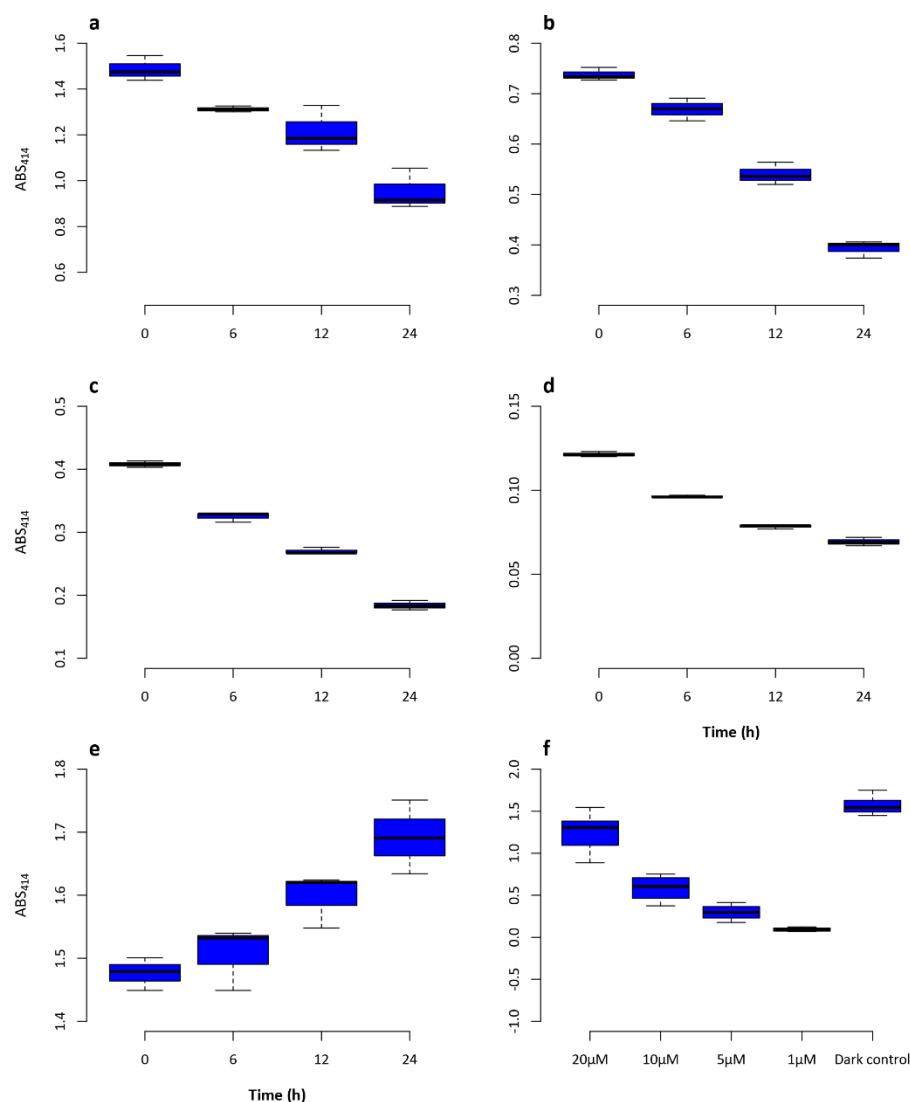


Figure A3.4: Boxplot showing homogeneity of variance in photobleaching experiment (ABS_{414}) using TPPS₄. Independent variables: time (a-f) and treatments (g). Figure: a = 20 μ M, b = 10 μ M, c = 5 μ M, d = 1 μ M, e = Light control (0 μ M), f = Dark control (20 μ M in the dark), g = all treatment over 24 h.

A3.2 PHOTOINACTIVATION EXPERIMENT

Table A3.5: Shapiro-Wilk normality test of each treatment in the photoinactivation experiment over 24 h of irradiation time using TMPyP porphyrin.

Treatment	W	p-value
20 μ M	0.63	2.13×10^{-09}
10 μ M	0.71	4.89×10^{-08}
5 μ M	0.85	4.01×10^{-05}
1 μ M	0.83	1.43×10^{-05}
Light control	0.67	8.33×10^{-09}
Dark control	0.63	1.92×10^{-09}

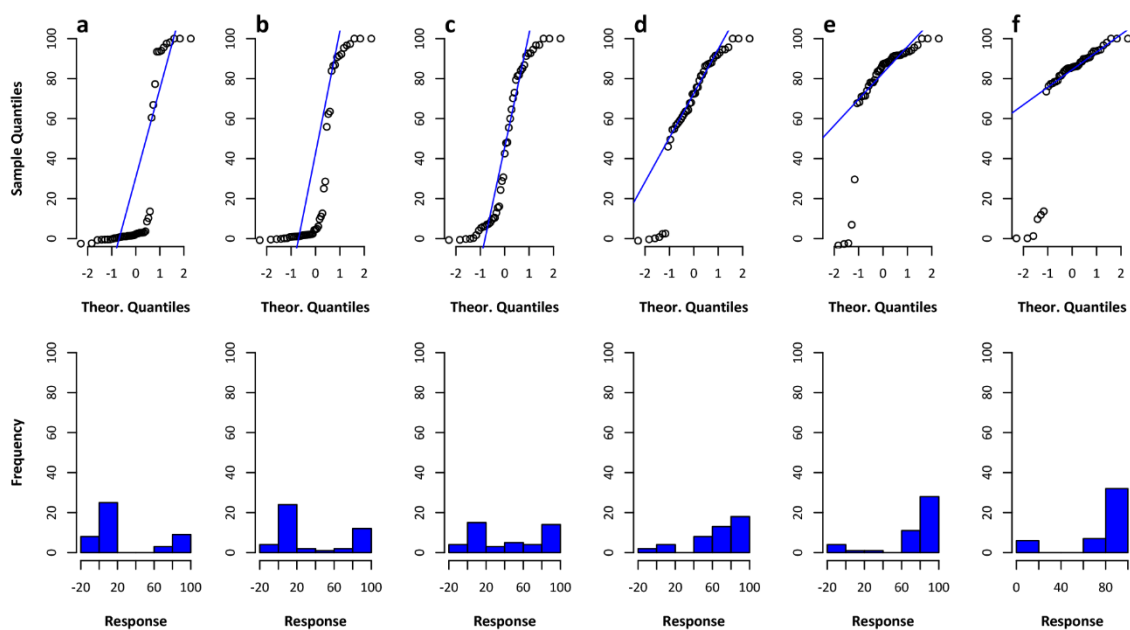
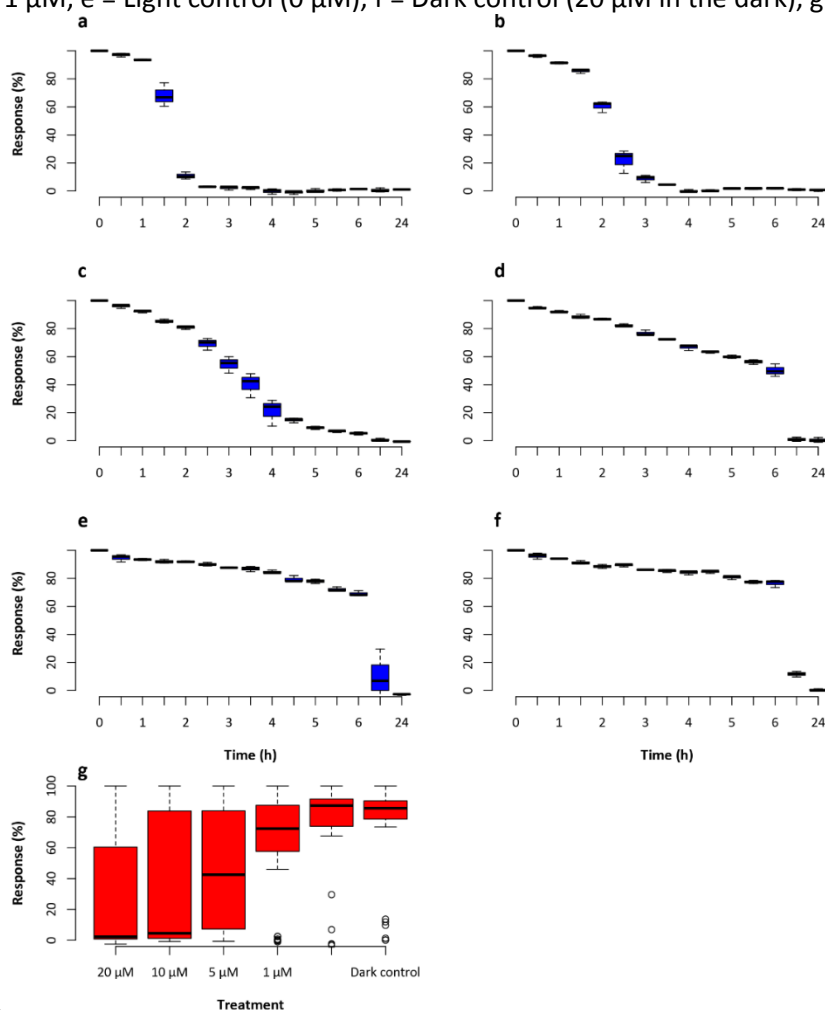


Figure A3.5: Graphic representation of normality distribution of each treatment in the photoinactivation experiment over 24 h of irradiation time using TMPyP. Top = Q-Q plots of sample quintiles against theoretical quantiles, Bottom = Histogram using frequency against response. Treatment: a = 20 μM , b = 10 μM , c = 5 μM , d = 1 μM , e = Light control (0 μM), f = Dark control (20 μM in the dark).

Table A3.6: Levene's Test results in photoinactivation experiment using TMPyP. Independent variables: Treatment (over 24 h) and time for each porphyrin concentration.

Treatments	Variable	Df	F-value	Pr(>F)
	Treatment	5	3.12	9.35×10^{-3}
20 μM	Time	14	4.57	2.38×10^{-4}
10 μM	Time	14	7.82	1.37×10^{-06}
5 μM	Time	14	5.28	6.69×10^{-05}
1 μM	Time	14	2.85	7.88×10^{-3}
Light control (0 μM)	Time	14	5.34	5.97×10^{-05}
Dark control (20 μM)	Time	14	2.37	2.31×10^{-2}

Figure A3.6: Boxplot showing homogeneity of variance in photoinactivation experiment using TMPyP. Independent variables: time (a-f) and treatments (g). Figure: a = 20 μ M, b = 10 μ M, c = 5 μ M, d = 1 μ M, e = Light control (0 μ M), f = Dark control (20 μ M in the dark), g = all treatment



over 24 h.

Table A3.7: Shapiro-Wilk normality test of each treatment in the photoinactivation experiment over 24 h of irradiation time using TPPS₄ porphyrin.

Treatment	W	p-value
20 μ M	0.63	2.13×10^{-09}
10 μ M	0.71	4.89×10^{-08}
5 μ M	0.85	4.01×10^{-05}
1 μ M	0.83	1.43×10^{-05}
Light control	0.67	8.33×10^{-09}
Dark control	0.63	1.92×10^{-09}

Figure A3.7: Graphic representation of normality distribution of each treatment in the photoinactivation experiment over 24 h of irradiation time using TPPS₄. Top = Q-Q plots of

sample quintiles against theoretical quantiles, Bottom = Histogram using frequency against response. Treatment: a = 20 μM , b = 10 μM , c = 5 μM , d = 1 μM , e = Light control (0 μM), f = Dark control (20 μM in the dark).

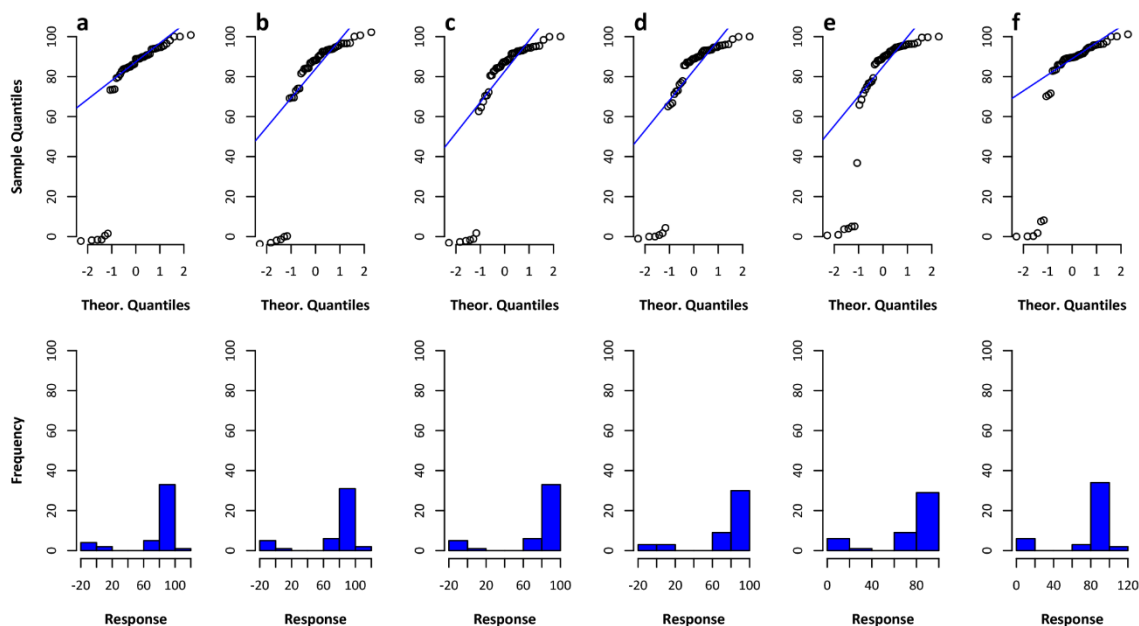


Table A3.8: Levene's Test results in photoinactivation experiment using TPPS₄. Independent variables: Treatment (over 24 h) and time for each porphyrin concentration.

Treatments	Variable	Df	F-value	Pr(>F)
	Treatment	5	0.03	0.99
20 μM	Time	14	2.05	4.91×10^{-2}
10 μM	Time	14	1.89	7.11×10^{-2}
5 μM	Time	14	3.04	5.20×10^{-3}
1 μM	Time	14	5.31	6.29×10^{-5}
Light control (0 μM)	Time	14	12.46	7.02×10^{-9}
Dark control (20 μM)	Time	14	6.09	1.74×10^{-5}

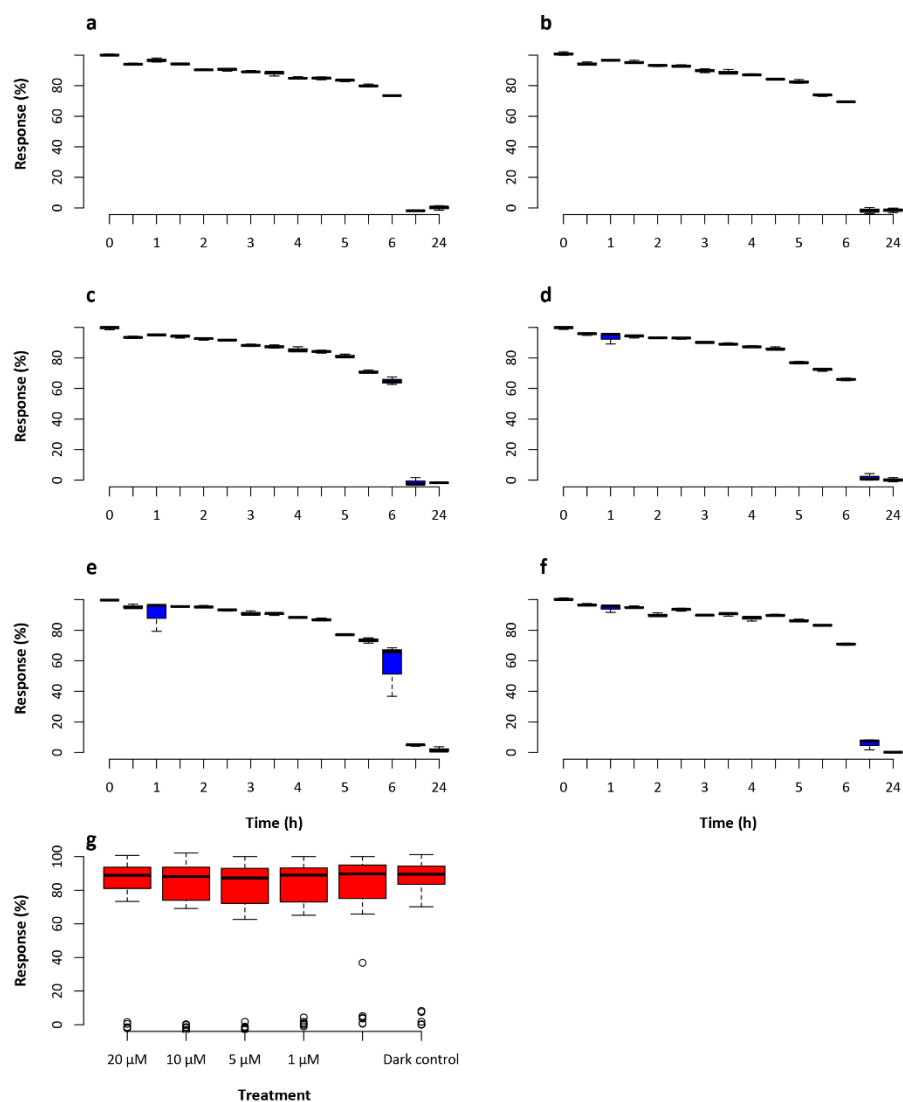


Figure A3.8: Boxplot showing homogeneity of variance in photoinactivation experiment using TPPS₄. Independent variables: time (a-f) and treatments (g). Figure: a = 20 μ M, b = 10 μ M, c = 5 μ M, d = 1 μ M, e = Light control (0 μ M), f = Dark control (20 μ M in the dark), g = all treatment over 24 h.

A3.3 ABSORBANCE (ABS₅₇₀) EXPERIMENT

Table A3.9: Shapiro-Wilk normality test for each treatment in the absorbance experiment (ABS₅₇₀) over 24 h of irradiation time using TMPyP porphyrin.

Treatment	W	p-value
20 μ M	0.96	8.39×10^{-02}
10 μ M	0.98	0.8
5 μ M	0.97	0.21
1 μ M	0.98	0.45
Light control	0.92	4.02×10^{-03}
Dark control	0.92	4.38×10^{-03}

Figure A3.9: Graphic representation of normality distribution of each treatment in the absorbance experiment (ABS₅₇₀) over 24 h of irradiation time using TMPyP. Top = Q-Q plots of sample quintiles against theoretical quantiles, Bottom = Histogram using frequency against response. Treatment: a = 20 μM, b = 10 μM, c = 5 μM, d = 1 μM, e = Light control (0 μM), f = Dark control (20 μM in the dark).

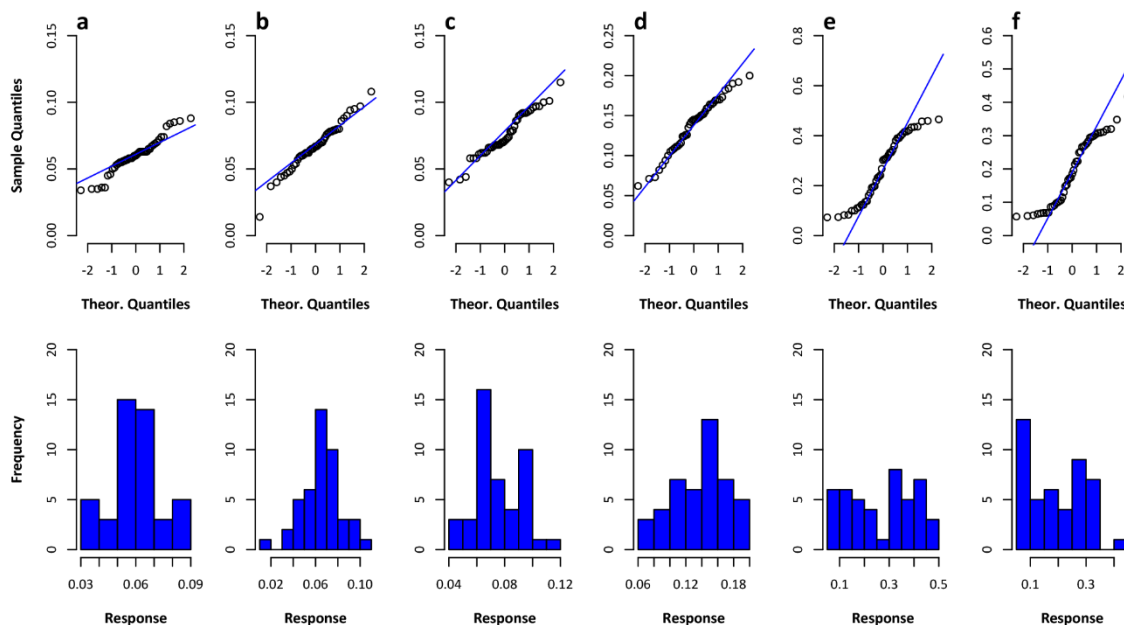


Table A3.10: Levene's Test results in ABS₅₇₀ experiment using TMPyP. Independent variables: Treatment (over 24 h) and time for each porphyrin concentration.

Treatments	Variable	Df	F-value	Pr(>F)
	Treatment	4	89.41	$< 2.22 \times 10^{-16}$
20 μM	Time	14	3.22	3.49×10^{-03}
10 μM	Time	14	6.94	4.78×10^{-06}
5 μM	Time	14	3.34	2.72×10^{-03}
1 μM	Time	14	3.57	1.70×10^{-03}
Light control (0 μM)	Time	14	3.48	2.02×10^{-03}
Dark control (20 μM)	Time	14	5.04	1.01×10^{-04}

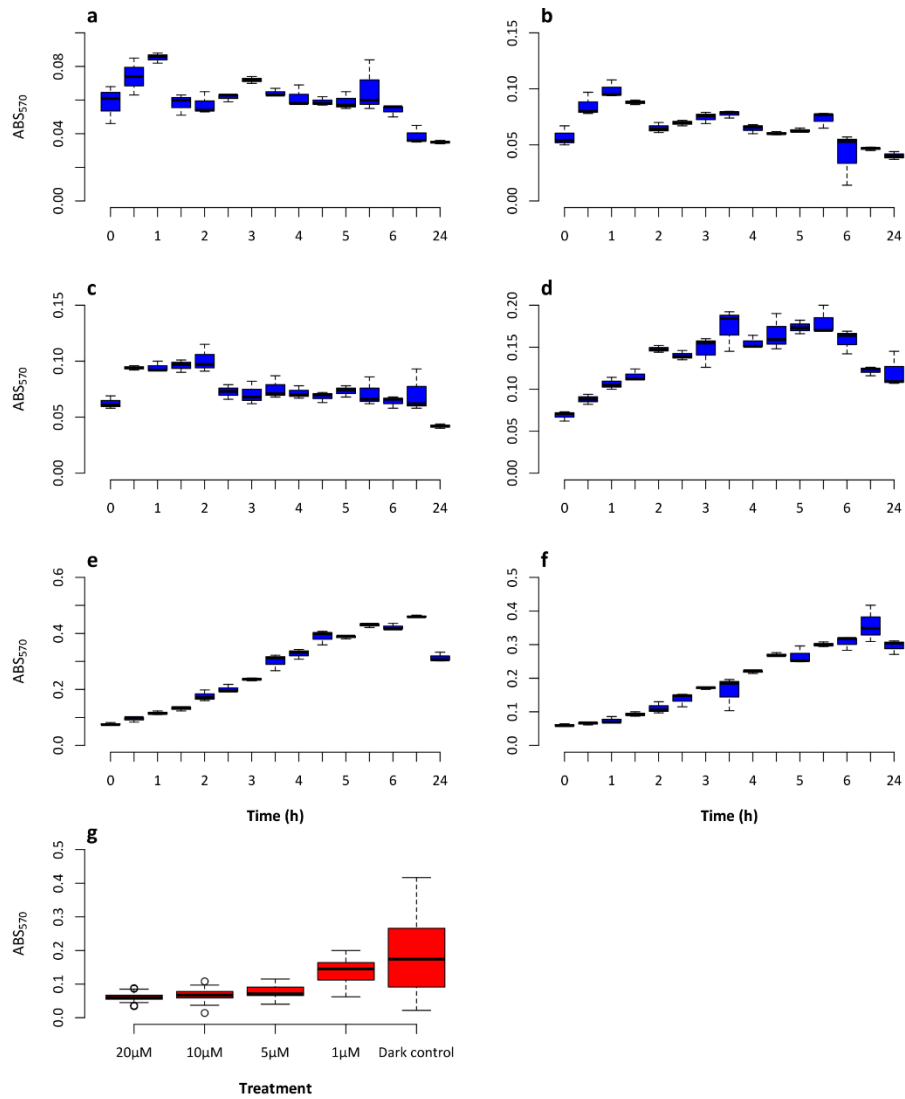


Figure A3.10: Boxplot showing homogeneity of variance in ABS_{570} experiment. Independent variables: time (a-f) and treatments (g). Figure: a = 20 μ M, b = 10 μ M, c = 5 μ M, d = 1 μ M, e = Light control (0 μ M), f = Dark control (20 μ M in the dark), g = all treatment over 24 h.

A4 APPENDIX CHAPTER 4

Table A4.1: Shapiro-Wilk normality test results of the time-course experiment.

Species	W	p-value
<i>C. muelleri</i>	0.75	1.76×10^{-15}
<i>T. lutea</i>	0.67	$< 2.20 \times 10^{-16}$
<i>N. oculata</i>	0.98	7.00×10^{-3}
<i>P. atomus</i>	0.72	$< 2.20 \times 10^{-16}$
<i>Te. chui</i>	0.71	$< 2.20 \times 10^{-16}$

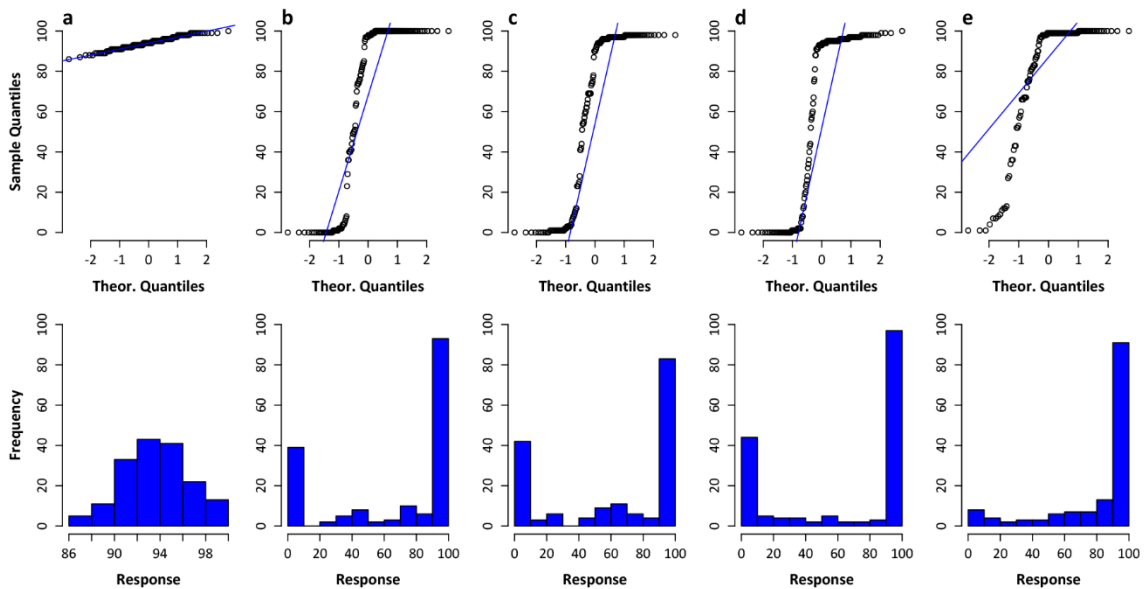


Figure A4.1: Graphic representation of normality distribution in time-course experiment. Top = Q-Q plots of sample quintiles against theoretical quantiles, Bottom = Histogram using frequency against response. *N. oculata*^a, *P. atomus*^b, *C. muelleri*^c, *Te. chui*^d, *T. lutea*^e.

Table A4.2: Levene's Test for Homogeneity of Variance using Time and Treatment as independent variables in time-course experiment.

Species		Df	F-value	Pr(>F)
<i>C. muelleri</i>	Time	6	40.22	$< 2.20 \times 10^{-16}$
	Treatment	7	9.13	1.80×10^{-9}
<i>T. lutea</i>	Time	5	6.76	1.14×10^{-5}
	Treatment	7	21.67	$< 2.2 \times 10^{-16}$
<i>N. oculata</i>	Time	6	4.59	2.52×10^{-4}
	Treatment	7	0.49	0.84
<i>P. atomus</i>	Time	6	18.15	4.95×10^{-16}
	Treatment	7	11.75	5.00×10^{-12}
<i>Te. chui</i>	Time	6	8.84	2.45×10^{-8}
	Treatment	7	5.30	1.80×10^{-5}

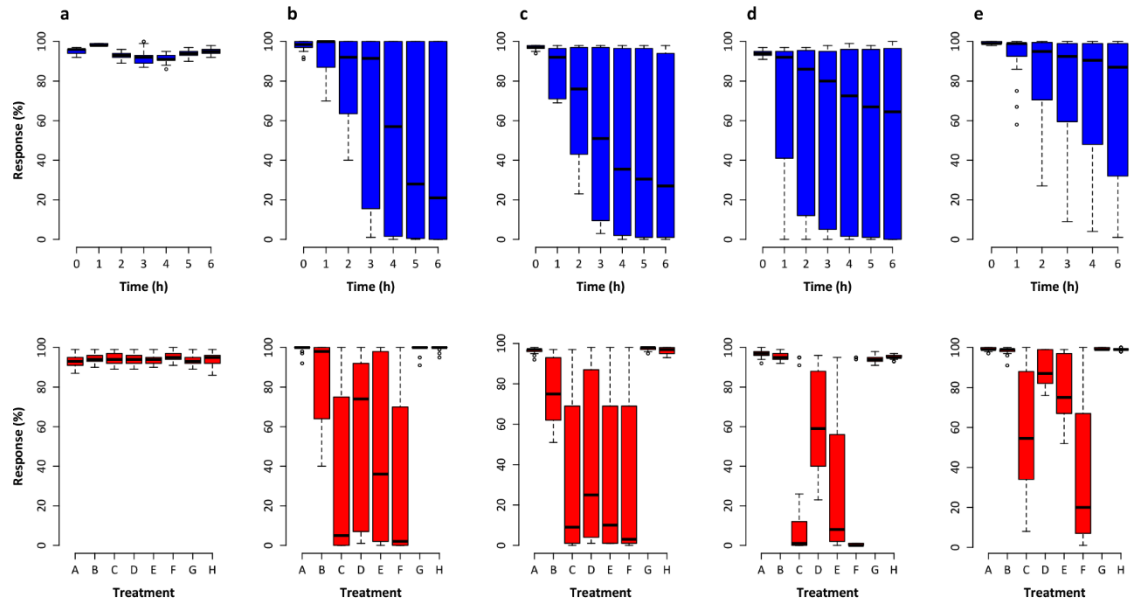


Figure A4.2: Boxplot of time-course experiment showing homogeneity of variance of *N. oculata*^a, *P. atomus*^b, *C. muelleri*^c, *Te. chui*^d, *T. lutea*^e. Top homogeneity of variance based on time. Bottom: homogeneity of variance based on treatments. Treatments: A = 0 μ M, B = 1 μ M, C = 20 μ M, D = 5 μ M, E = 10 μ M, F = 50 μ M, G = Dark control (0 μ M) and H = Dark control (50 μ M).

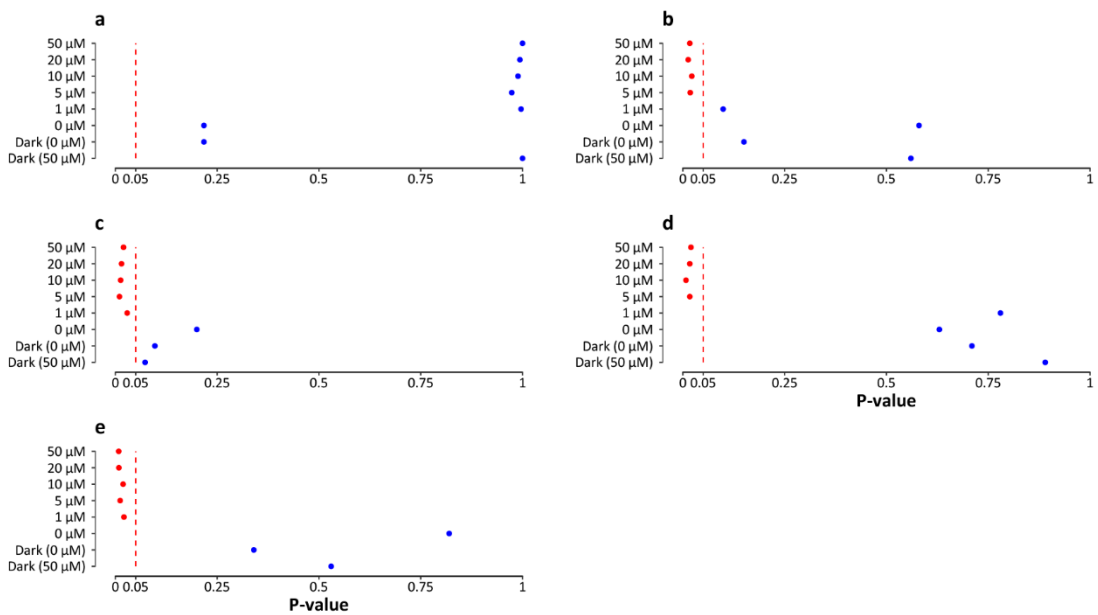


Figure A4.3: Graphic representation of p-values for each treatment after comparing start (0 h) and end (6 h) of the time-course experiment in *N. oculata*^a, *P. atomus*^b, *C. muelleri*^c, *Te. chui*^d, *T. lutea*^e. Red dots represent significant difference, while blue dots represent no significant difference. Significant level = 0.05 (Dashed line).

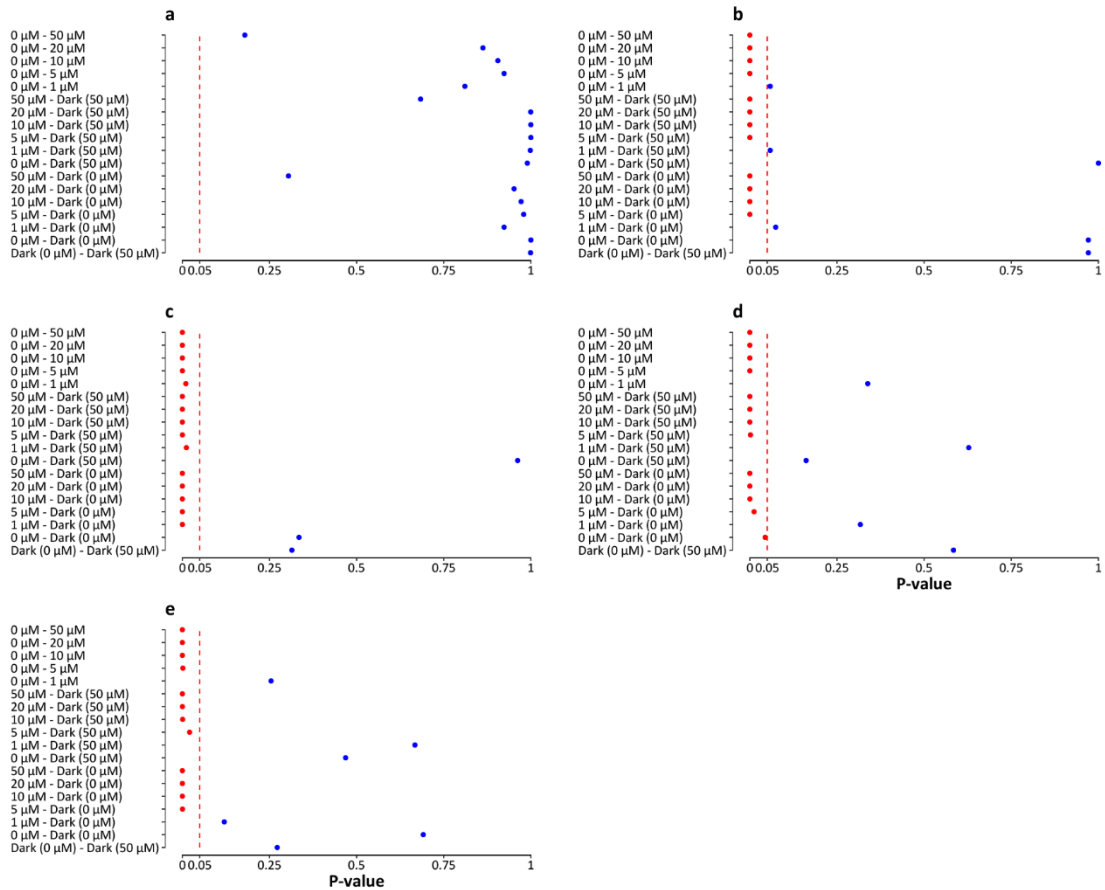


Figure A4.4: Graphic representation of p-values after pairwise comparison of each treatment in time-course experiment of *N. oculata*^a, *P. atomus*^b, *C. muelleri*^c, *Te. chui*^d, *T. lutea*^e. Red dots represent significant difference, while blue dots represent no significant difference. Significant level = 0.05 (Dashed line).

Table A4.3: Shapiro-Wilk normality test results of the dose-response at time 6 h.

Species	W	p-value
<i>C. muelleri</i>	0.68	4.51x10 ⁻⁰⁵
<i>T. lutea</i>	0.82	3.13x10 ⁻⁰³
<i>N. oculata</i>	0.98	0.89
<i>P. atomus</i>	0.68	4.59x10 ⁻⁰⁵
<i>Te. chui</i>	0.72	1.35x10 ⁻⁰⁴

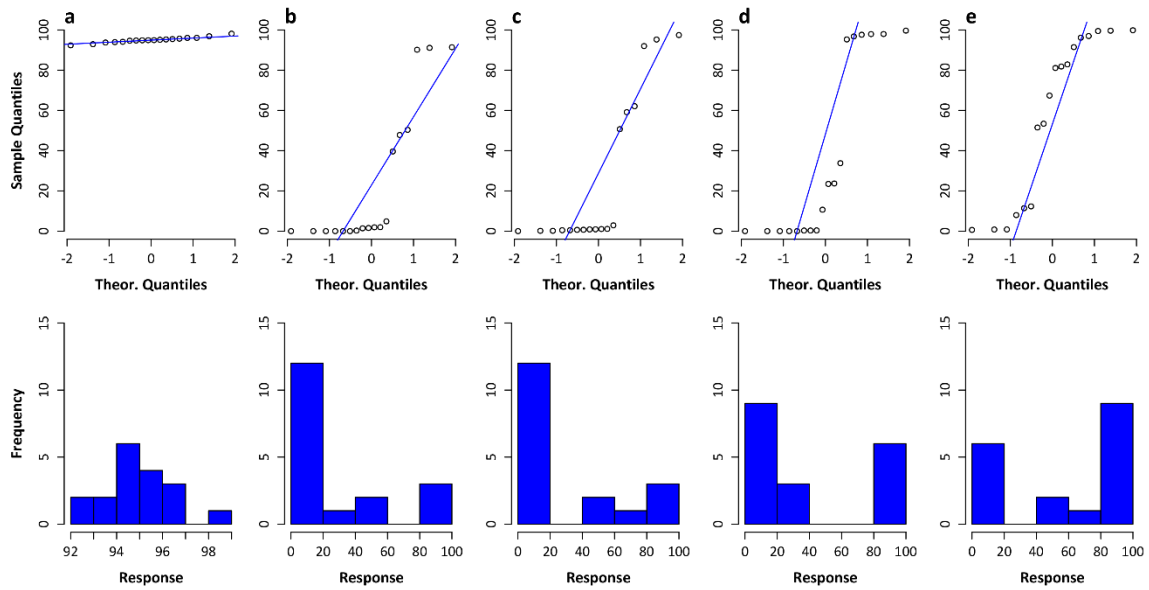


Figure A4.5: Graphic representation of normality distribution at time 6-h of *N. oculata*^a, *P. atomus*^b, *C. muelleri*^c, *Te. chui*^d, *T. lutea*^e. Top = Q-Q plots of sample quintiles against theoretical quantiles, Bottom = Histogram using frequency against response.

Table A4.4: Levene's Test for Homogeneity of Variance using Treatment at time 6 h as variable.

Species	Df	F-value	Pr(>F)
<i>C. muelleri</i>	5	6.31	4.28x10 ⁻⁰³
<i>T. lutea</i>	5	9.78	6.53x10 ⁻⁰⁴
<i>N. oculata</i>	5	1.35	0.31
<i>P. atomus</i>	5	7.92	1.67x10 ⁻⁰³
<i>Te. chui</i>	5	9.53	7.34x10 ⁻⁰⁴

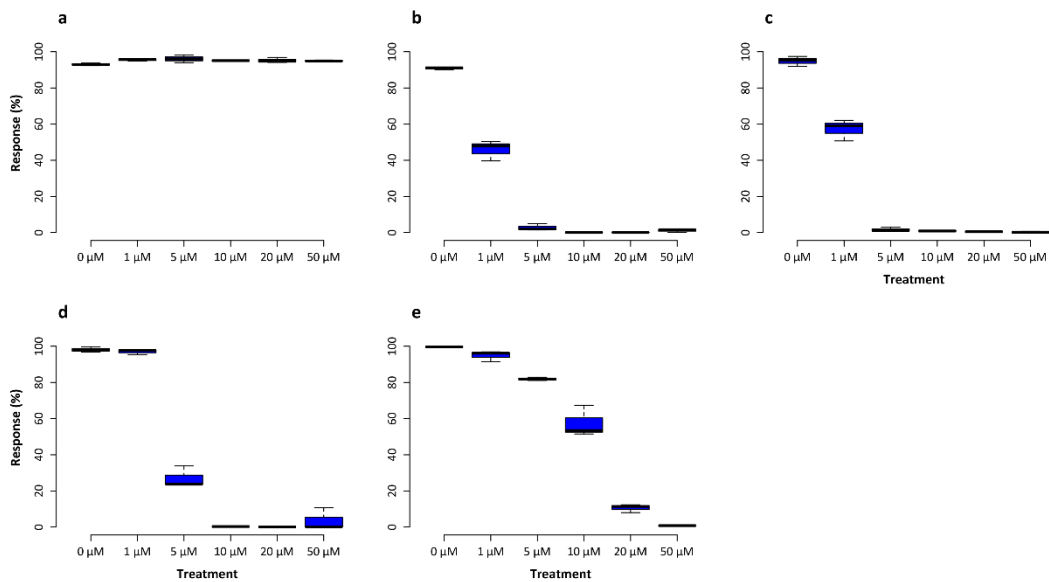


Figure A4.6: Boxplot showing homogeneity of variance of *N. oculata*^a, *P. atomus*^b, *C. muelleri*^c, *Te. chui*^d, *T. lutea*^e of treatments at time 6-h.

A5 APPENDIX CHAPTER 5

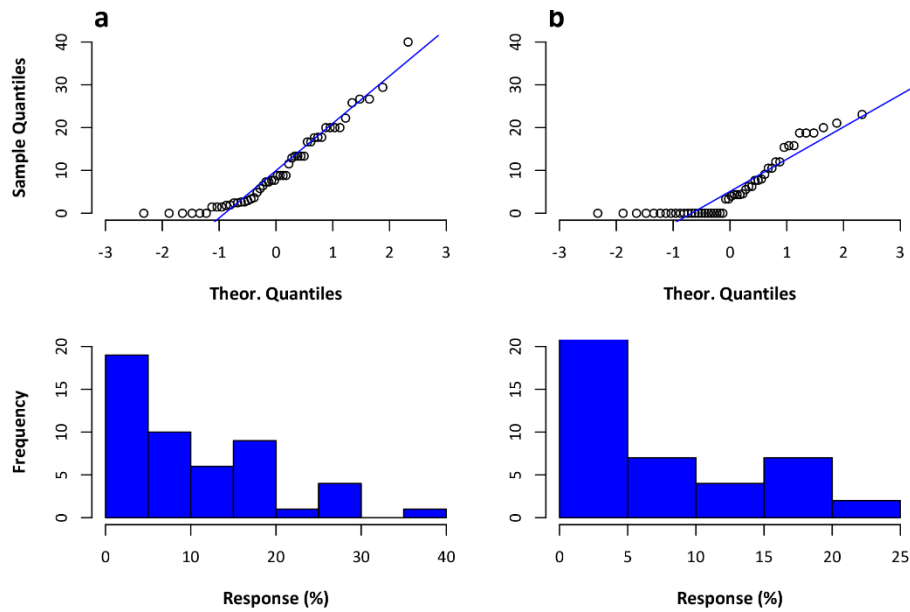


Figure A5.1: Graphic representation of normality distribution at time 6-h of magnetic (a) and unmodified (b) cysts. Top = Q-Q plots of sample quintiles against theoretical quantiles, Bottom = Histogram using frequency against response (%). Shapiro-Wilk normality test: magnetic cysts ($W = 0.90$, $p = 6.74 \times 10^{-04}$); unmodified cysts ($W = 0.80$, $p = 9.03 \times 10^{-07}$).

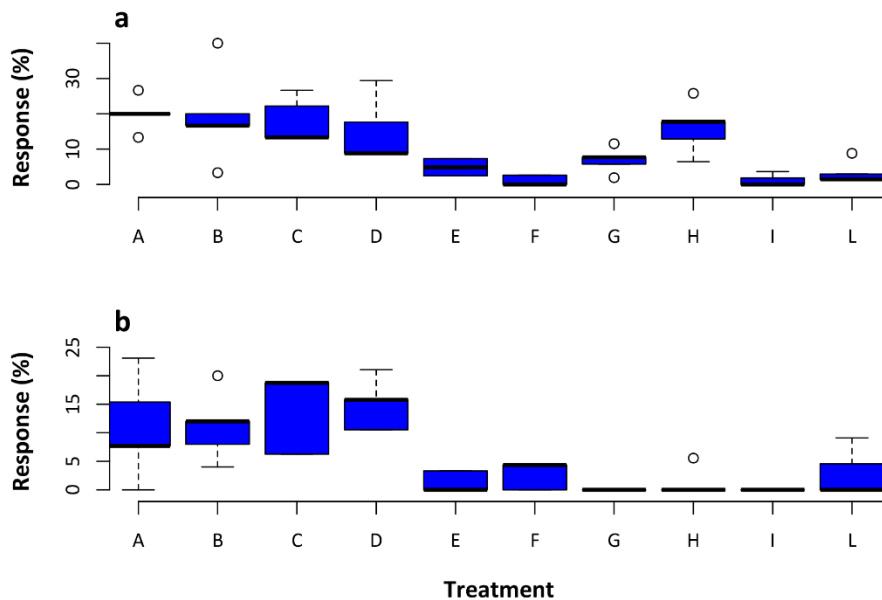


Figure A5.2: Boxplot showing homogeneity of variance at time 6-h of magnetic (a) and unmodified (b) cysts. Treatments A:G light expose, H:L dark exposed. Treatments= A = 50 μM, B = 20 μM, C = 10 μM, D = 5 μM, E = 1 μM, F = 0 μM + DMSO (Light control), G = 0 μM no DMSO (Light control), H = 0 μM + DMSO (Dark control), I = 0 μM no DMSO (Dark control) and L = 50 μM (Dark control). Levene test results: Magnetic cysts [Df = 9, F value = 2.20, $Pr(>F) = 0.042$]; Norml cysts [Df = 6, F value = 6.07, $Pr(>F) = 1.15 \times 10^{-4}$].