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Sensitivity of live microalgal aquaculture feed to singlet oxygen-based photodynamic therapy

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Abstract

Highly nutritional microalgal species are extensively used in aquaculture as live feedstock. Due to difficulties in maintaining microalgae in axenic conditions, they represent a potential pathogen carrier and disease vector in aquaculture ponds. Photodynamic therapy (PDT) via singlet oxygen ($^1O_2$) production is a promising sterilization technique in aquaculture. Here, we report on the sensitivity of aquaculture-relevant microalgae towards $^1O_2$ generated by the cationic photosensitizer TMPyP. Possible PDT sterilization protocols of contaminated microalgae cultures were evaluated using the luminescent bacterium *Vibrio campbellii* ISO7 as a model aquaculture pathogen. Species-specific sensitivity of microalgae to TMPyP-mediated PDT was demonstrated and found to be strongly influenced by the nature and architecture of their respective cell wall. While cytotoxicity was not evident against *Nannochloropsis oculata*, toxicity of $^1O_2$ was dose-, time- and light activation-dependent against *Tisochrysis lutea*, *Tetraselmis chui*, *Chaetoceros muelleri* and *Picochlorum atomus*. The $^1O_2$-resilient *N. oculata* was sterilized when incubated under light in the presence of *V. campbellii* ISO7 (up to 10^7 CFU mL^{-1}) and 20 μM TMPyP; hence, TMPyP-based PDT sterilization of *N. oculata* could be suitable for aquaculture hatcheries. This study also suggests that PDT using cationic porphyrins such as TMPyP holds potential as an algicidal treatment in aquaria and aquaculture systems (but more research using opportunistic and toxic species is needed for confirmation).

Keywords Microalgae · Photoinactivation · Sterilization · Aquaculture live feed · Photodynamic antimicrobial chemotherapy

Introduction

Marine microalgae are at the base of most marine food webs and 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) are among the reasons for the growing interest in commercial production of microalgae (Salvesen et al. 2000; Natrah et al. 2014; Zhang et al. 2014). Marine microalgae are 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 potentially biofuel production (Borowitzka and Moheimani 2010; Fon Sing et al. 2013; Islam et al. 2013; Pandey et al. 2014; Liu and Chen 2016). Today, the demand for microalgal biomass is primarily driven by its use as live feed in aquaculture of a wide range of organisms including molluscs, crustaceans and fish

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(Salvesen et al. 2000; Zhang et al. 2014) which feed on algae either directly or indirectly via zooplankton enrichment.

Microalgae cultures represent a biosecurity threat to aquaculture operations, as they are potential vectors of pathogenic bacteria (Salvesen et al. 1999) and are generally added continuously or at regular intervals, especially as live feed in hatcheries. While recurrent introduction of bacteria that are closely associated with microalgae in a mutualistic relationship might have positive effects on the bacterial community of the larval system (Salvesen et al. 1999; Salvesen et al. 2000), the introduction of opportunistic pathogens into high-nutrient environments needs to be avoided, as it can lead to disease outbreaks with severe financial repercussions (Salvesen et al. 1999; Natrah et al. 2014; Pintado et al. 2014; Unnithan et al. 2014). For example, ideal growth conditions for *Vibrio alginolyticus*, a potential aquaculture animal pathogen, occur in cultures of the widely used microalga *Chaetoceros muelleri* (Gomez-Gil et al. 2002) and up to 10^3 CFU mL^{-1} of *Vibrio* bacteria were counted in cultures of *Pavlova lutheri* (Salvesen et al. 2000). Consequently, intensive aquaculture operations allocate large resources to controlling bacterial loads in microalgal cultures, with new technologies being continuously developed (Pintado et al. 2014).

A wide array of sterilization techniques have been developed specifically for the reduction of bacteria in aquaculture; however, each technology has its own limitations. The production of singlet oxygen ($^1O_2$) and reactive oxygen species (ROS) during photodynamic therapy (PDT) is considered an innovative sterilization method for the aquaculture industry, as it creates a toxic environment for microorganisms including pathogenic bacteria that lack a specific $^1O_2$ detoxification system (Maisch 2015). Generally, bacteria might express enzymes such as superoxide dismutase, catalase and peroxidase (ROS detoxification system) that increase cell survival when exposed to sub-lethal ROS dosages. These ROS-detoxifying enzymes are, however, less efficient in combating ROS damage afflicted externally (i.e. cell wall and cell membrane) (Maisch 2015). Therefore, bacterial cells are usually unable to tolerate ROS and $^1O_2$ build-up in the environment. Furthermore, superoxide dismutase, catalase and peroxidase are not able to quench $^1O_2$ (Wainwright and Crossley 2004) and are inactivated by $^1O_2$ (Kim et al. 2001). Photosynthetic microorganisms, on the other hand, have specific defence mechanisms to protect themselves from possible damage by internal ROS and $^1O_2$. The algal anti-oxidant defence system is composed of both anti-oxidant enzymes and non-enzymatic strategies. Enzymatic defences include superoxide dismutase, catalase, guaiacol peroxidase and enzymes of the ascorbate-glutathione cycle, ascorbate peroxidase (monodehydroascorbate reductase, dehydroascorbate reductase and glutathione reductase), fulfilling the same ROS detoxification function as in animal, plant and bacterial cells, while non-enzymatic components are cellular oxido-reduction buffers (ascorbate and glutathione) and compounds (tocopherols, phenols and carotenoids), the latter being capable of quenching $^1O_2$ (Noctor and Foyer 1998; Glaeser et al. 2011; Sharma et al. 2012; Vatansever et al. 2013). Few studies have been conducted to confirm the superior tolerance of photosynthetic organisms to ROS and $^1O_2$ build-up. For instance, Drábková et al. (2007) showed that microalgae and cyanobacteria are both sensitive to $^1O_2$ and Pohl et al. (2015) showed that green algae are sensitive to cationic photosensitizers (PS) and some anionic ones. However, the current paucity of evidence on microalgal resistance to PDT makes it difficult to estimate how effective these techniques could potentially be for aquaculture microalgal feed sterilization purposes.

The aims of the present study were (1) to test the toxicity of ROS (mainly $^1O_2$) generated by the cationic PS TMPyP during PDT 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) to investigate if PDT can be used to disinfect a PDT-resilient microalgae culture seeded with the naturally luminescent model pathogen *V. campbellii* ISO7 (Malara et al. 2017a). This study represents the first documentation of porphyrin-generated $^1O_2$ toxicity on aquaculture phytoplankton species.

Materials and methods

Toxicity test

Algae strain and growth condition

Marine microalgae were obtained from the North Queensland Algae Identification Facility (NQAIF, James Cook University; *Tisochrysis lutea*, *Nannochloropsis oculata*, *Tetraselmis chui* and *Picochlorum atomus*) and from the Australian Institute of Marine Science (AIMS, Townsville, Australia; *Chaetoceros muelleri*). The strain identity and cell size range of each species are reported in Table 1.

Microalgal species were selected based on (1) their use in aquaculture hatcheries as live feed (FAO 2007; Brown and Blackburn 2013), (2) their cell wall composition and (3) their fatty acid profiles and lipid content.

Microalgae were cultured in either f/2 medium (Guillard 1975) (*Chaetoceros muelleri*) or in modified L1 liquid medium (Guillard and Hargraves 1993) (all other strains). Media were sterile-filtered rather than autoclaved to avoid precipitation and high background noise in the subsequent flow cytometer analysis. Seawater was filtered through a 0.22-μm Durapore membrane filter (Millipore, Australia) and other culture media components were filtered through a 0.22-μm

Table 1 List of microalgae strains including species name, culture collection accession number and cell size

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

[a] Previously described as *Isochrysis affinis galbana* (Bendif et al. 2013)

syringe filter (Minisart high-flow membrane filter, Sartorius Stedim Biotech, Germany).

Photosensitizer

The PS used in this work was the cationic porphyrin TMPyP described by Malara et al. (2017a). A stock solution was prepared by diluting the PS in 100% dimethyl sulfoxide (DMSO) to a concentration of 10 mM (stock solution). Aliquots (1.5 mL) of stock solution were stored at 3–4 °C covered in aluminium foil to shield the PS from any light exposure. At the start of each experiment, the PS stock solution was diluted in L1 or f/2 medium as appropriate to the concentrations described in Fig. 1. The molecular structure and full absorbance spectra (350 and 750 nm) of TMPyP at concentrations between 0 and 20 μM are presented in Figure S1.

Microalgae viability evaluation

Microalgae viability was evaluated using a flow cytometer (InCyte, benchtop Merck Millipore, Australia) and the dye propidium iodide (PI) (Aldrich, Australia). PI does not pass through cell membranes and therefore binds nucleic acids only when cells are damaged (dead) (Darzynkiewicz et al. 1994; Berney et al. 2007). Details on PI fluorescence excitation and emission spectra can be found at the manufacturer's website (www.invitrogen.com). Propidium iodide was diluted in phosphate-buffered saline (PBS) at a concentration of 3.5 mg PI in 1 mL PBS and stored at 4 °C in the dark, unless stated otherwise, PI final concentration in each sample to stain cells was approximately 7×10^{-4} mg mL^{-1}. 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-florescence and PI (Olson and Chisholm 1986; Van Bleijswijk and Veldhuis 1995), the yellow channel (yellow florescence (YF)) was used to detect the PI signal. Before each experiment and for each microalgal species, the protocol was optimized as follows: (1) one aliquot (4 mL) of fresh microalgal culture was split into two 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), and (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 (Fig. 2).

Sensitivity of microalgae to singlet oxygen-based PDT

Considering the difference in cell size of the five microalgal species used, the inoculum size was normalized for each species using dry weight rather than cell number at final dry weight concentration of ~0.04 g L^{-1}. Table S1 shows the equations used to transform cell number to dry weight (g L^{-1}) for each organism. Microalgal cultures were maintained in L1 medium, with the exception of *C. muelleri* cultures, which were maintained in f/2 medium. Final concentrations of the porphyrin TMPyP were 50, 20, 10, 5 and 1 μM with the 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 microalgal species using controls with DMSO (0.5%) but no porphyrin (light control; Fig. 1).

Samples and controls (2.5 mL, 3 independent replicates) were placed in 12-well tissue culture plates (product # 353043, Beckton Dickinson, USA). The light control (0 μM TMPyP) contained 0.5% DMSO to monitor potential solvent toxicity and produce baseline response data for the microalgae (Fig. 1). 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 a negative effect on the microalgae population (0 μM).

Samples were incubated for 20 min in the dark under continuous movement to promote porphyrin-binding to the cells

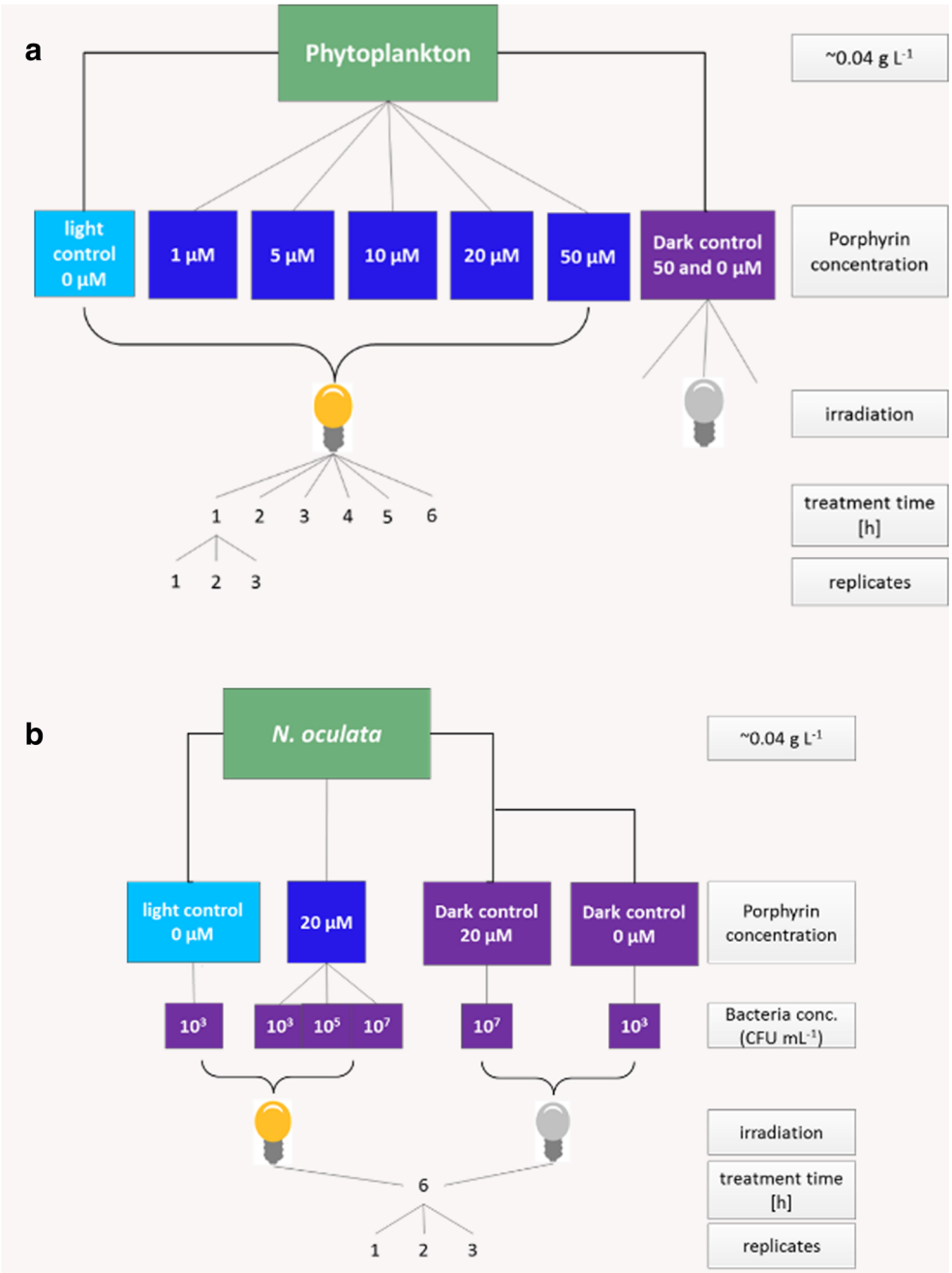


Fig. 1 **a** The experimental design used in dose–response and time-course experiments. **b** The experimental design used for disinfection of a seeded microalgae culture

and were subsequently exposed to constant light for 6 h as described by Malara et al. 2017a. Well-plates were placed on an orbital shaker (150 rpm) to keep samples homogenized.

Unless stated otherwise (Table S1), 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. Each replicate and dilution was vacuum-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). An aliquot (200 µL) was used to determine cell number using the flow cytometer (see above for details). Immediately after filtration, filters were washed with ammonium formate (0.5 M, pH 8.0, adjusted with 1 M NaOH) at a ratio of 2:1 (*v*/*v*; filtered culture: ammonium formate). Filters were 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, after cooling to room temperature in a desiccator). The weight of the filtered algae was calculated as the average difference in filter weight before and after the filtration process, and the specific weight was found by dividing the algal weight by the volume (mL) of algae filtered.

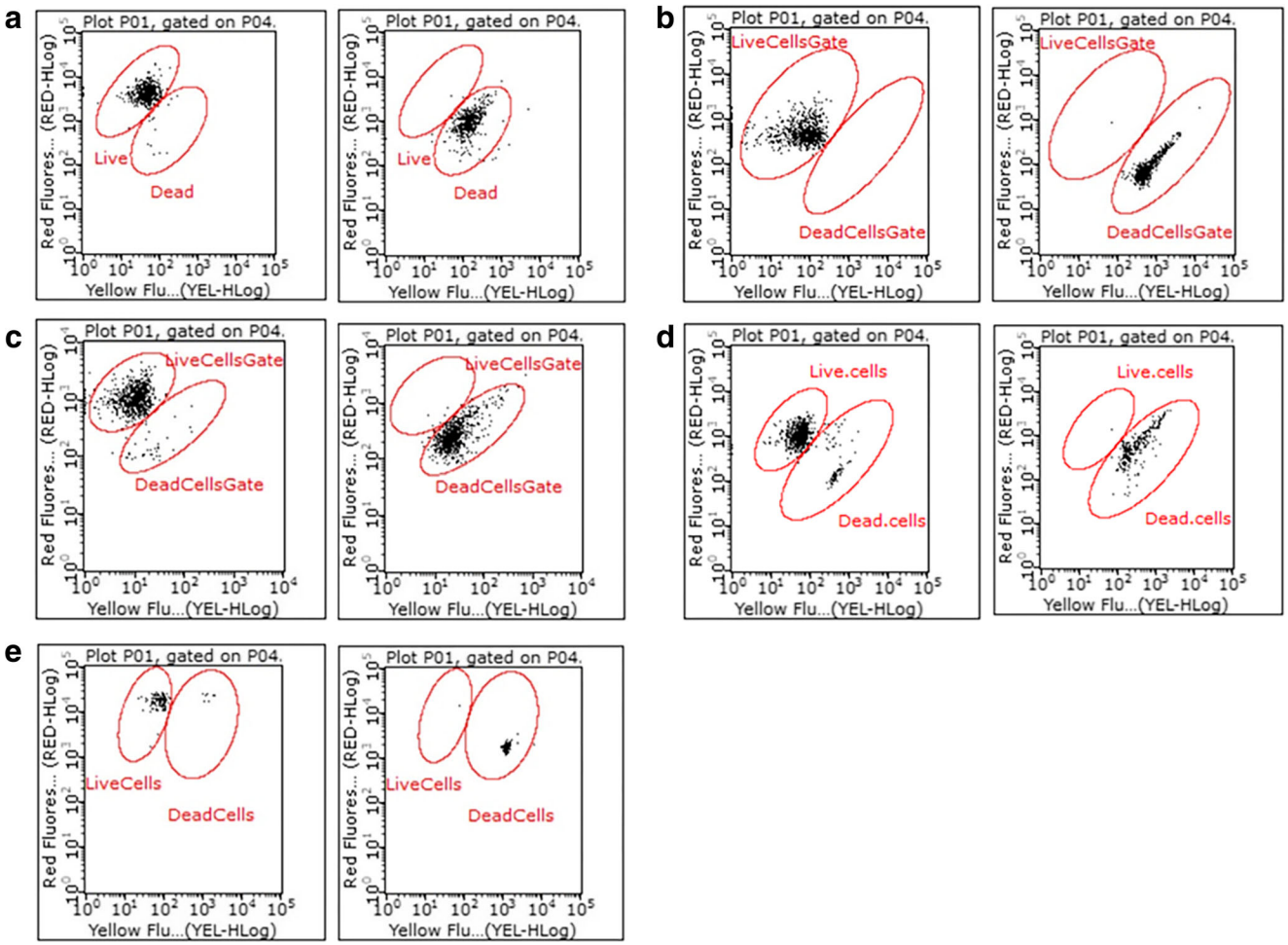


Fig. 2 Flow cytometer determination of live and dead (by heating) cells of *C. muelleri* (**a**), *T. lutea* (**b**), *N. oculata* (**c**), *P. atomus* (**d**) and *Te. chui* (**e**) using the dye PI

PDT disinfection of microalgal culture seeded with a model bacterial pathogen

The model bacterium used to simulate bacterial contamination was the naturally luminescent *Vibrio campbellii* ISO7, which previously showed virulence against *Penaeus monodon* when injected (Malara et al. (2017a). 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 and enumerated as described previously (Malara et al. 2017a).

The TMPyP-resilient microalga *N. oculata* was used in this experiment with growth conditions as described above. The experimental design is presented in Fig. 1b. The *N. oculata* was diluted to a final concentration of ~0.04 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, 3 independent replicates) were added to a 12-well tissue culture plate (product # 353043, Beckton Dickinson, NJ, USA) and exposed to light irradiation as described above. At the start and end of the experiment (times 0 and 6 h), samples were taken for CFU counts and species-specific most probable number (MPN) determination. The number of CFUs on LA was determined by spread-plating (0.1 mL, $n = 3$, 28 °C for 24 h) and luminescent colonies were documented using an image acquisition system (Fusion FX, Vilber Lourmat, France). A strategy combing MPN analysis with a multiplex PCR that can discriminate between the closely related species *V. campbellii*, *Vibrio harveyi*, *Vibrio owensii* and *Vibrio rotiferianus* (Cano-Gomez et al. 2015) was used to detect *V. campbellii* ISO7 in mixed cultures with *N. oculata* before and after PDT treatment. Samples taken for MPN determination (1.5 mL) were centrifuged at 10,621×*g* for 15 min. The resulting pellets were washed with sterile L1 medium, followed by resuspension in 5 mL of peptone salt solution (3% NaCl, 0.1% bacteriological peptone). For each sample, three dilution series (1:10) were prepared in alkaline peptone water (mAPW: 1% bacteriological peptone, 3% NaCl, pH 8.4, final volume = 10 mL) and incubated for 16–20 h at 28 °C. The DNA from harvested cells was released and amplified as previously reported (Malara et al. 2017a).

Statistical analysis

Statistical analysis and graphs were performed using R-studio version 0.99.896 (RStudio Team 2015), setting the significance level α to 0.05 and testing for normality (Figure S2) and homogeneity of variance (Figure S3; *Rcdrm* package, v.2.3) using the Shapiro–Wilk test and Levene's test, respectively (Fox 2005, 2007).

For each microalgal species, treatment effects (porphyrin concentration) and time effects were investigated using either ANOVA (Chambers et al. 1992) or the Kruskal–Wallis test (Hollander and Wolfe 1973; R Core Team 2015) when ANOVA assumption were violated (*stats* package v.3.2.5). When time or treatment effects were observed, 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's honestly significant differences test (Miller 1981) was used for ANOVA-based statistics and a Dunn rank test after a Kruskal–Wallis 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 PDT treatment after 6 h of irradiation. The live cell proportion for each phytoplankton population (time 6 h) 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 establish the relative potency (RP) (a measure for quantifying the strength) of one microalgal species over another (Ritz et al. 2015).

Results

Toxicity test

In general, porphyrin treatment in the absence of light (dark 50 μM) and dark exposure in the absence of the porphyrin TMPyP (dark 0 μM) (Fig. 1a) had no significant effect ($p > 0.05$) on population size (live, dead cells) of any of the five microalgae tested (Figure S4). This proved that the porphyrin itself was not cytotoxic over the 6 h exposure period and that the dark condition itself did not result in death of the microalgae. Furthermore, the light source used did not photodamage any of the microalgal species, as control samples without porphyrin (0 μM) in the light and in the dark showed no significant difference (p value > 0.05; Figure S4).

In the presence of light, the live cell concentration of four of the five microalgae tested (*P. atomus*, *C. muelleri*, *Te. chui* and *T. lutea*) were negatively affected by both TMPyP concentration and exposure time (Figs. 3, S4 and S5; Tables S2 and S3). Of these, *T. lutea* and *Te. chui* showed no significant differences to control groups at 1 μM TMPyP (p value > 0.05; Figure S5), demonstrating their ability to cope with low amounts of PDT-produced ROS.

Only *N. oculata* showed no significant treatment effect on population size (p value > 0.05; Fig. 3e, Table S2). For this alga, the ANOVA suggested an effect of time (Table S3), and there was a small dip in the population size after 2 h exposure (Fig. 3e). However, there was no significant difference in population size between the start and the end of the experiment (time 0 and 6 h) (Figure S4).

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; Fig. 4, Table 2). In particular, sensitivity to TMPyP-produced ROS (mainly $^1O_2$) decreased in the following order; *P. atomus* ≥ *C. muelleri* > *Te. chui* > *T. lutea* > *N. oculata* (no effect), making the chlorophytes (*P. atomus*, *Te. chui*) and bacillariophyte (*C. muelleri*) the most sensitive species. This was confirmed by pairwise species comparison using relative potency calculations based on the ratio of 6 h-IC_{50} concentrations (Table S4). Furthermore, all porphyrin treatments for these three microalgal species were significantly different to light controls (0 μM; p value < 0.05, Figure S4), supporting the conclusion that they are highly sensitive to TMPyP-produced ROS (mainly $^1O_2$) (Fig. 4). *Tetraselmis chui* and *T. lutea* showed an intermediate response: these species did not show mortality when incubated with 1 and 5 μM of TMPyP but populations exposed to higher TMPyP concentrations in the light were significantly different from controls ($p < 0.05$, Figure S5, Table 3: 6 h-$IC_{50} = \sim 4$; Table S4: RP > 1).

Disinfection of contaminated culture

In this experiment, a culture of the TMPyP-PDT-tolerant microalga *N. oculata* was seeded with different concentrations of the model bacterium *V. campbellii* ISO7. Six hours of irradiation in the presence of 20 μM 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 S6). Plates containing CFUs showed a high diversity of colonies therefore photographic documentation of luminescence was necessary. 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 S6). This unusual behaviour might be explained by respiration of *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 and enumeration of *V. campbellii* present in the mixed culture with *N. oculata*. Samples collected at the start of the experiment (Figs. 5a and S7a) and control samples

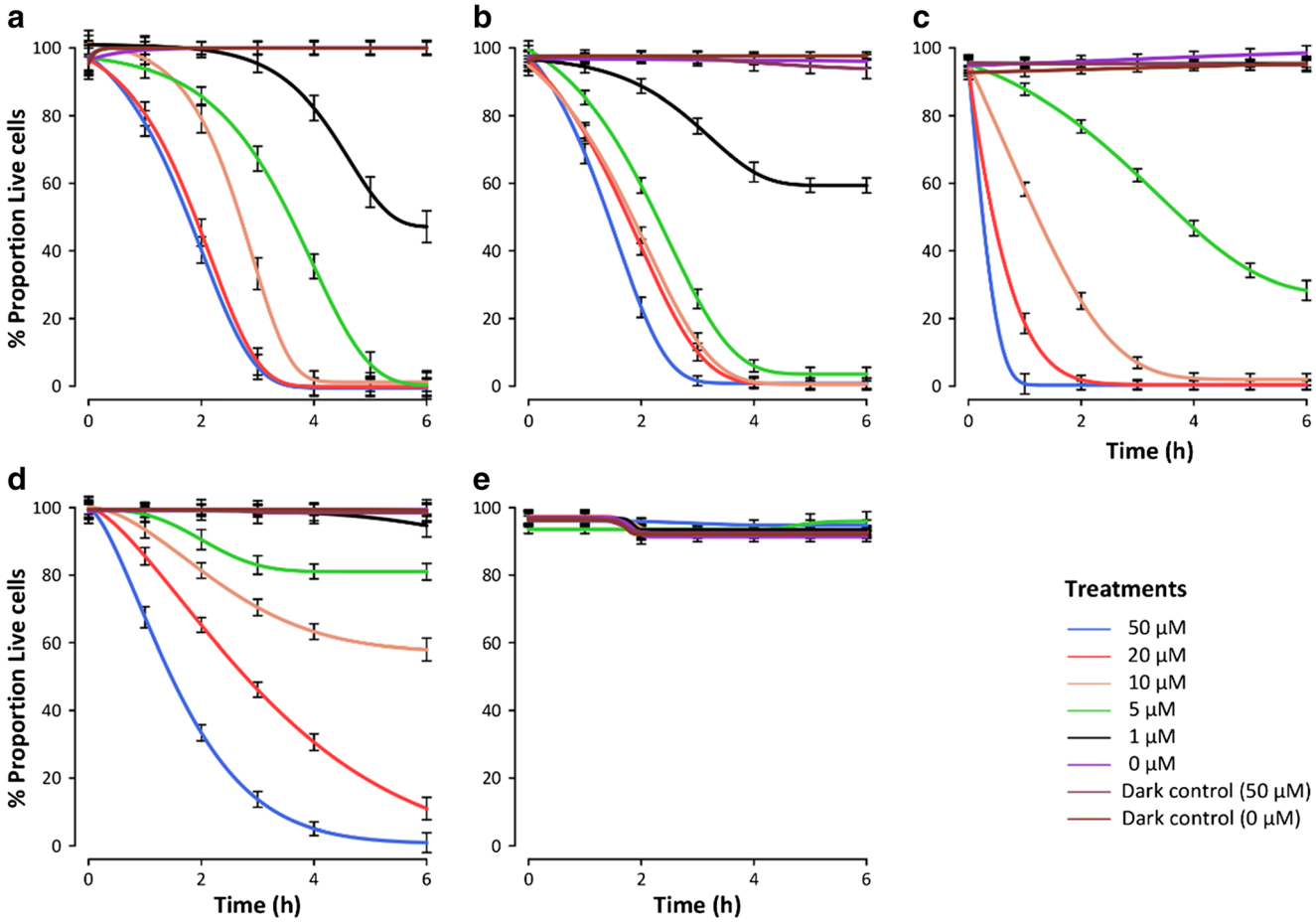


Fig. 3 Effect of exposure time and TMPyP concentration on population size (% of live cells) of *P. atomus* (**a**), *C. muelleri* (**b**), *Te. chui* (**c**), *T. lutea* (**d**) and *N. oculata* (**e**). For each species, the best fitting model of all possible candidates was selected based on AIC values

(start and end of experiment; Figs. 5b and S7a, b) produced growth in culture tubes and multiplex PCR confirmed the presence of the model bacterium *V. campbellii*. In contrast, samples irradiated for 6 h in the presence of TMPyP showed no visible regrowth (data not shown) and produced no PCR amplification product (Fig. 5b).

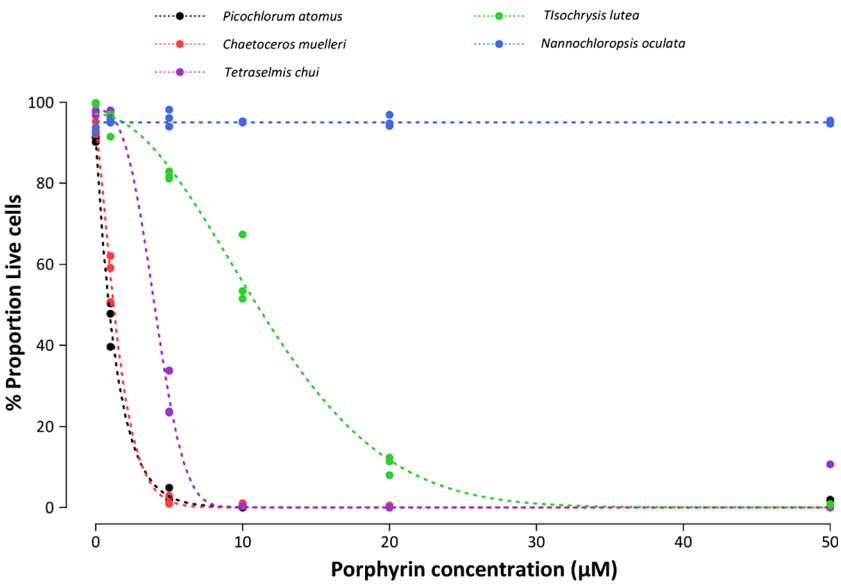


Fig. 4 Dose–response curve of *N. oculata*, *P. atomus*, *C. muelleri*, *Te. chui* and *T. lutea* at time 6 h. The model was obtained using the R-studio (version 0.99.01) and "drc" package

Table 2 TMPyP concentration [μM] that reduced the population size by 50% after 6 h of irradiation (6 h-IC_{50} ± SE) for each microalgal species in a dose–response model. *N. oculata* returned infinite values and was not included

Species	6 h-IC_{50}
P. atomus	1.02 ± 0.06
C. muelleri	1.27 ± 0.07
Te. chui	4.04 ± 0.39
T. lutea	11.21 ± 0.28

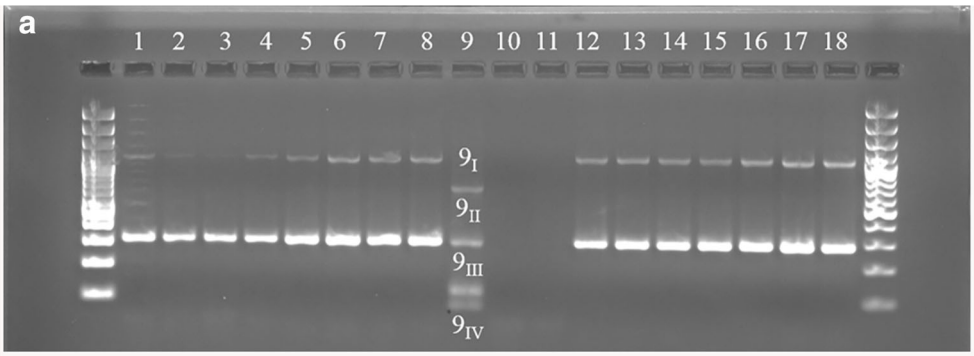
Discussion

Photodynamic antimicrobial therapy is considered a non-selective method to kill organisms due to the production of $^1O_2$ as the main ROS (Carey 1992; Jori and Brown 2004; Magaraggia et al. 2006; Alves et al. 2011). Singlet oxygen is a reactive species of oxygen able to oxidize cellular components (proteins, lipids, nucleic acids, carbohydrates) in all organisms (Skovsen et al. 2005), but effectiveness is influenced by treatment time, light intensity, concentration

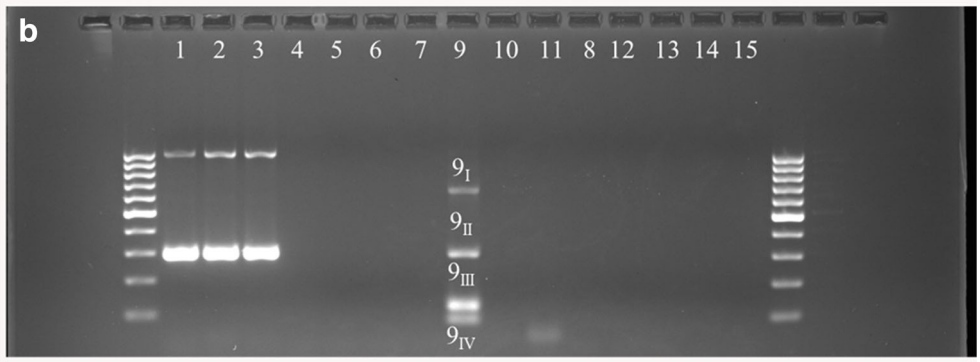
Table 3 Cell surface organization and biochemical composition of the genera *Tetraselmis*, *Isochrysis*, *Chaetoceros*, *Picochlorum* and *Nannochloropsis*

Cell surface Organization	Phylum/Class	Species	Cell wall biochemical composition	References
Theca (non-mineralized) Flagellar scales	Chlorophyta/Prasinophyceae	*Tetraselmis tetrahele*; *T. striata*	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
Cell wall	Chlorophyta/Trebouxiophyceae	*Chlorella* sp.; *Trebouxia* sp.	Cellulose; algaenans (aliphatic polymethylenic polymer chains) conjugated with amides and N-alkyl-substituted pyrroles (*Chlorella* sp.) or β-galactofurans (*Trebouxia*)	Brown and Elfman 1983; Domozych et al. 2012
		Picochlorum atomus	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	*Isochrysis* sp.; *I. galbana*	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	*Chaetoceros fusiformis*; *C. affinis*; *C. curvicetus*; *C. decipiens*; *C. debilis*; *C. sociales*	Cell wall silica associated with proteins (frustulins, pleurfins, 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	*Nannochloropsis gaditana*; *N. oculata*; *N. salina*	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

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* Xylo



Treatment	Lane number				
	1, 2, 3	4, 5, 6	7, 8, 12	13, 14, 15	16, 17, 18
Time [h]	0	0	0	0	0
Light	Yes	Yes	Yes	Yes	No
MPN dilution	No	No	No	No	No
V. campbellii ISO7 [CFU mL^{-1}]	10^3	10^5	10^7	10^3	10^7
TMPyP	+	+	+	-	+



Treatment	Lane number			
	1, 2, 3	4, 5, 6	7, 8, 12	13, 14, 15
Time [h]	6	6	6	6
Light	No	Yes	Yes	Yes
MPN dilution	1:10^7	No	No	No
V. campbellii ISO7 [CFU mL^{-1}]	10^7	10^3	10^5	10^7
TMPyP	+	+	+	+

Fig. 5 Species-specific MPN-PCR detection of the model bacterium *V. campbellii* ISO7 in seeded *N. oculata* cultures before and after PDT with 20 μM TMPyP. **a** Samples (lanes 1–8, 12) and controls (lanes 13–18) at time 0. **b** Dark control (lanes 1–3) and samples (lanes 4–8, 12–15) at time 6 h. For each gel, lane 9 shows the positive controls (9_I = *Vibrio rotiferianus*, 9_{II} = *Vibrio campbellii*, 9_{III} = *Vibrio harveyi*, 9_{IV} = *Vibrio owensii*), lane 10 shows the negative control (*Vibrio fortis*) and lane 11 shows the blank reaction (Milli-Q water). Gels showing additional controls are presented in Figure S7

and type of PS used and the ability of target organisms to withstand the toxic effect of $^1O_2$.

No cell death occurred in our dark controls with 50 μM of the porphyrin TMPyP for any of the tested microalgae, confirming that the cationic porphyrin TMPyP per se was not cytotoxic to the selected species. This finding is consistent with previous reports on porphyrins and *Vibrio* spp. (Malara et al. 2017a, b). In other studies, the photosensitizing dyes methylene blue (6 μM) and nuclear fast red (10 μM) incubated with the green alga *Chlorella vulgaris* (McCullagh and Robertson 2006b) and methylene blue (6 μM) incubated with the cyanobacterium *Synechococcus leopoliensis* (McCullagh and Robertson 2006a) were reported to be not toxic in the dark in the presence or absence of hydrogen peroxide (H_2O_2). In contrast, Pohl et al. (2015) determined that the anionic corroles $SbCor^-$ and $PCor^-$ reduced biomass by 5%, while dark controls were not affected. They concluded that exposure to corroles and light induced intracellular photo-oxidation of chromophore (bleaching), concluding that PS might have an effect on photosynthetic organelles.

Recently, fluorescence localization studies during PDT in *Chlorella fusca* var. *vacuolata* by Bornhütter et al. (2016)

demonstrated that the cationic porphyrin TMPyP accumulate intracellularly while the cationic corrole $PCor^+$ (5,10,15-tris-(1-methylpyridinium-2-yl)corrolato-(trans-dihydroxo)phosphorus(V)) was observed attached to the cell wall. This suggests that TMPyP is actively taken up and/or produces $^1O_2$ (and other ROS) in proximity to the cell wall causing initial damage and then enters the periplasmatic space, similar to what is proposed for bacteria (Stojiljkovic et al. 1999; Jori and Coppellotti 2007). Consequently, the observed species-specific sensitivity of TMPyP-generated $^1O_2$ against microalgae in our work can be explained by differences in the biochemical nature and organization of their cell coverings leading to differences in the interaction of TMPyP with the microalgal surfaces (Table 3).

The highest sensitivity to PDT was seen for the chlorophyte *P. atomus* and the bacillariophyte *C. muelleri*. Sensitivity of chlorophytes is consistent with previous studies investigating toxicity of phthalocyanines, tetraphenol porphyrine, methylene blue and cationic and anionic corroles towards the chlorophytes *Pseudokirchneriella subcapitata*, *Stichococcus bacillaris*, *Chlorella fusca* var. *vacuolata*, *Scenedesmus quadricauda* and *Chlorella kessleri* and the cyanobacteria *Synechococcus nidulans*, *Microcystis incerta* and *Anabaena* sp. (Drábková et al. 2007; Jancula et al. 2008; Pohl et al. 2015; Bornhütter et al. 2016).

Picochlorum atomus (syn. *Nannochloris atomus*) is a unicellular coccoid chlorophyte belonging to the Trebuxiophyceae. The cell walls of the Trebouxiophyceae typically consist of cellulose, algaenan and structural proteins (Table 3), whose disulphide linkages require reduction to weaken the cell wall (Porra 2011). Since the cell wall of *Picochlorum* is thin (Table 3), it is possible that oxidation of structurally important proteins could allow TMPyP-produced ROS (mainly $^1O_2$) to oxidize plasma membrane constituents, e.g. fatty acids, leading to the observed high vulnerability to PDT. 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).

The bacillariophyte *C. 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 (Reinke 1984; Lemmermann 1898). 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 *C. muelleri*, but based on information obtained for several *Chaetoceros* species (Gügi et al. 2015), the cationic porphyrin is highly likely to complex tightly with frustule components, generating $^1O_2$ and other ROS in very close proximity to the cell, potentially destabilizing the thin and fragile frustule and plasma membrane phospholipids of the organism.

Intermediate sensitivity to PDT was seen for the chlorophyte *Tetraselmis chui* and the haptophyte *Tisochrysis lutea*. As a chlorophyte in the family Prasinophyceae, the theca of *Te. chui* is characterized by dominance of 2-keto sugar acids over neutral sugars (Table 3). 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 $^1O_2$ production in close proximity to the plasma membrane, explaining the observed sensitivity of this organism to TMPyP-generated ROS (mainly $^1O_2$) 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-mineralized organic scales bound together by acidic polysaccharides which are also thought to connect the cellulose microfibrils (Table 3). We hypothesize 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 ROS (mainly $^1O_2$) and the longer time frames required for reduction in population size to occur.

The microalga *N. oculata* was found to be resilient to PDT with the cationic porphyrin TMPyP. *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 3) (Geldin et al. 1999; Scholz et al. 2014). Our data suggests that the hydrophobic straight-chain hydrocarbons that comprise the outer layer of the *Nannochloropsis* cell wall make the organism resilient to oxidation by the cationic porphyrin TMPyP-produced $^1O_2$.

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 (Watanabe et al. 2005; Cho et al. 2015) and, as phytohormones, macro- and micronutrients produced by bacteria increase algae growth rate (Croft et al. 2005; Bolch et al. 2011; Teplitski and Rajamani 2011; Kazamia et al. 2012; Kuo and Lin 2013; Kim et al. 2014; Ramanan et al. 2016). In addition, creating and maintaining axenic microalgae cultures on a large scale is regarded as challenging and often unrealistic (Pintado et al. 2014). To avoid the introduction of potential pathogenic bacteria

via algal cultures, especially in hatchery sectors where larvae are more sensitive to infections, it would be advantageous if bacteria-free microalgae cultures could be produced immediately before feeding (Vadstein 1997). Hence, new strategies are needed to minimize the presence of potential pathogenic bacteria and ideally create axenic "ready to feed" algae-inoculi in aquaculture.

Different methods are currently 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 (Wiedeman et al. 1964; Bowyer and Skerman 1968; Hoshaw and Rosowski 1973; Carmichael and Gorham 1974; Sykora et al. 1980; Brown 1982; Divan and Schnoes 1982; Connell and Cattolico 1996; Jorquera et al. 2002; Yim and Lee 2004; Watanabe et al. 2005; Gasulla et al. 2010; Suga et al. 2011). While antibiotics is the most commonly used method (Cottrell and Suttle 1993; Connell and Cattolico 1996; Cho et al. 2013), it is difficult, not economic and time-consuming (Yim and Lee 2004) and can be toxic to microalgae cells (Sensen et al. 1993; Yim and Lee 2004; Youn and Hur 2007; Seoane et al. 2014). Most importantly, antibiotics in aquaculture should be avoided due to the potential accumulation in the environment, resistance of microorganisms, consumer reservations and human health concerns (Peggy and Francis-Floyd 1996; Romalde 2002; Arijo et al. 2005; Alves et al. 2011; Bermúdez-Almada and Espinosa-Plascencia 2012).

The TMPyP-PDT-resistant strain *N. oculata* was used in sterilization experiments to optimize conditions needed to create a gnotobiotic algae culture. Six hours of irradiation using 20 μM of TMPyP generated enough ROS (mainly $^1O_2$) to result in 100% lethality of the model bacterium *V. campbellii* ISO7 in mixed microalga-bacteria incubation experiments. In addition, the DNA of the model bacterium was not detected using molecular techniques; therefore, as DNA is not the primary target of $^1O_2$ (Bonnett and Berenbaum 2007; Maisch 2007; Tavares et al. 2011; Dosselli et al. 2012; Sperandio et al. 2013; Almeida et al. 2015), the absence of nucleic acids in multiplex PCR indicates irreversible damage to the bacterial cells. The method used here appears to be more practical for producing axenic *Nannochloropsis* spp. cultures than the method proposed by Cho et al. (2013) combining ultrasonication with fluorescence-activated cell sorting (FACS) and micropicking, In contrast, the method proposed by Cho et al. (2013) is more suitable for sterilizing cultures of green microalgae, e.g. *C. vulgaris*, *C. sorokiniana* and *Scenedesmus* sp., which were found to be sensitive to the used TMPyP-based PDT protocol.

Conclusions

TMPyP-generated ROS (mainly $^1O_2$) showed species-specific toxicity against microalgal cells. Their ability to resist the TMPyP-generated $^1O_2$ was strongly influenced by the nature and architecture of their respective cell walls, which influences the sterical and conformational interaction of the PS with the cell wall and hence toxicity of $^1O_2$. Of the algal species tested here, only *N. oculata* was suited for TMPyP-based PDT sterilization to produce bacteria-free algal feeds. The approach can potentially be used for other algal species if time and dosage combinations can be found that eradicate bacterial pathogens without damaging the algal cells. Our results suggests that TMPyP-based PDT could be used as an algicidal treatment in aquaria and aquaculture similarly to AquaFrin (porphyrin) (Schrader et al. 2010), but more research using opportunistic and toxic species is needed for confirmation.

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