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Axenisation of oleaginous microalgal cultures via anoxic photosensitisation

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ABSTRACT

Growing interest in sustainable food and biofuel research has necessitated high quality axenic oleaginous microalgal strains. Unfortunately, most strains available in culture banks contain commensal microbes such as bacteria and the default decontamination method involves antibiotic treatment which has begun to exacerbate the emergence of antibiotic resistance. To overcome this problem, anoxic photosensitisation was investigated as an alternate approach.

Four oleaginous microalgal species (*Tetradesmus obliquus, Desmodesmus armatus*, *Chlorella vulgaris* and *Nannochloropsis limnetica*) were incubated in varying concentrations of Rose Bengal (0 μM, 1 μM, 3 μM or 9 μM) either in normal (oxic) or anoxic conditions, for 72 h under light (8.85 ± 0.4 W/m^2) in a specially designed heterotrophic growth complex (HGC) medium, followed by 72 h in standard Bold's Basal Medium (BBM). Commonly used antibiotics-based protocol was used as the control method. Post treatment, cell numbers and percentage populations were counted with Flow Cytometry, and viability was tested using standard plating methods using BBM and LB. Additionally, the contaminating microbes in the cultures were profiled using 16Ss rRNA sequencing.

Anoxic conditions were able to significantly decrease bacterial content, albeit with an equally detrimental effect on the microalgal population. Although the responses differed between the microalgae, anoxic incubation along with Rose Bengal at 3 μM was able to completely decontaminate *N. limnetica* and *C. vulgaris*, while *D. armatus* and *T. obliquus* could be decontaminated with an additional streak-plating step. None of the cultures could be decontaminated using antibiotics treatment.

These results suggest that axenisation of microalgal cultures was largely due to anoxy, that was synergistically enhanced by Rose Bengal at a concentration of ≥3 μM.

1. Introduction

With growing impetus to achieve sustainable climate targets through the generation of sustainable fuels, food, and carbon capture (Goal 7, [1]), microalgal research has not only seen a significant boost in research interest, but more resources are now being channelled towards studying their molecular mechanisms. This has raised the demand on high quality oleaginous strains; many of which are sensitive to contaminating microbes in the culture [2]. Many microalgae of interest; though easily available in culture banks, are xenic and contain bacteria or other microbial contaminants that interfere with further upstream research work. Consequently, axenisation / decontamination procedures remains an active topic of research. The most commonly employed first line of treatment to tackle bacterial loads is with antibiotics [3] that consequently, has resulted in resistance development owing to indiscriminate use [4]. While newer methods have been developed with a higher focus on physical separation through microfluidics [5–7], and advances in flow cytometry allows for more precise sorting of cells in larger volumes of culture [8], many of these methods become unreliable when working with bacterial-microalgal associations that are physically

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attached. Bacteria can lodge on the microalgal surface and become very difficult to remove. Using vigorous physical methods or harsh antibiotic use can become deleterious to the microalgae itself. Moreover, while many protocols call for a combination of chemical and physical methods to attain axenic cultures, such procedures quickly become protracted, expensive, and can significantly hamper the survivability of the microalgal culture.

The work described in this publication attempts to investigate the combined physico-chemical treatment on contaminated microalgal cultures through anoxy and photosensitisation to achieve axenic cultures. Photosensitisation through the use Rose Bengal appears as a lucrative method of decontamination against bacterial and viral particles [9]. Rose Bengal (RB) is a halogenated xanthene dye that is widely recognized for its potent photosensitization properties. Rose Bengal has previously been used to inactivate a broad spectrum of pathogens, including bacteria, fungi, and viruses using photodynamic antimicrobial chemotherapy (PACT), where the dye is exposed to visible light to produce reactive singlet oxygen ($^1O_2$) species that damage microbial membranes, proteins, and nucleic acids [10]. This mechanism has been effective against drug-resistant bacteria, providing a promising alternative to conventional antibiotics. Studies have also demonstrated Rose Bengal's efficacy in biofilm disruption, a significant advantage in addressing persistent infections [11]. Bacteria generally do not posses the necessary pathways to mitigate the production of free oxygen radicals that are generated when Rose Bengal is exposed to light in an aqueous medium [12]. Moreover, being a xanthene derivative, Rose Bengal possess anti-bacterial properties even in the absence of light and has been used in formulation with chloramphenicol to isolate fungi from mixed cultures [13]. Likewise, removal of oxygen from cultures has been observed to greatly aid decontamination procedures [14]. While this is generally achieved through flushing the growing microalgal culture with CO_2 or N_2., the pH requires regular adjustment.

The experimental setup described herein sought was to leverage the ability of microalgae to not only survive under anoxic conditions through the production of its own oxygen via photosynthesis, but also utilise its photosynthetic apparatus to expunge excess oxygen radicals generated from the Rose Bengal dye and selectively culture them free of other contaminating organisms.

2. Material and methods

Water used in the experiments was purified via a Milli-Q® purification system. The experiment was performed in biological triplicate. Sterile techniques were performed in a Laminar flow hood (Hearus, Ireland) with all equipment doused in 70 % (*v*/v) denatured alcohol, and exposed to UV light for at least 10 min prior to commencement of experiments. Anaerobic / anoxic conditions were maintained in an anaerobic chamber (Whitney Biosciences).

Dye Stock Solution (DSS, 10 mM, 10 mL) was prepared using Rose Bengal Dye (101.7 mg, Thermo Fisher Scientific, India) and sterilised by filtering through a sterile 0.22 μm filter (Millex GV, PVDF membrane).

2.1. Media

Incubation of microalgae in Rose Bengal was carried out in a heterotrophic growth complex (HGC, 200 mL, pH 7.2): Tryptone (10 g/L; GBiosciences; St. Louis; USA), Yeast extract (5 g/L; UltraPure; Thermo Scientific, USA), Coral Pro Salt (1 g/L; Red Sea, Israel), and pyruvic acid (20 mg/L; Merck, Germany) to supplement growth even during the continuous dark phases. For anoxic conditions, 100 mL medium was autoclaved and then placed in the anaerobic chamber and allowed to equilibrate under constant stirring for five days. For aerobic incubation conditions, 100 mL of the medium was autoclaved and used without any further modifications.

Mother cultures and post-treatment culturing was carried out in standard 3 N + BBM [15]. Similar to HGC, anaerobic 3 N + BBM (100 mL) was prepared by autoclaving the medium and placing it in the anaerobic chamber for five days and allowing equilibration for five days under constant stirring.

2.2. Microalgal species

Xenic mother cultures (900 mL) of *Desmodesmus armatus* (from Prof, Yu's lab, [16]), *Tetradesmus obliquus* (CCAP 276/10), *Chlorella vulgaris* (CCAP 211/19), and *Nannochloropsis limnetica* (SAG 18.99) cultures were maintained in ambient (21 °C to 25 °C) temperature conditions under constant shaking (80 rpm) with a 14 h: 10 h light cycle at 8.85 ± 0.4 W/m^2 brightness (Barrina T5 LED Grow Lights, Full spectrum, USA).

2.3. Anoxic treatment conditions

Under aseptic conditions in a laminar hood, the xenic microalgal mother culture (50 mL) was drawn in sterile centrifuge tubes (Falcon®, BD), centrifuged at 3000 ×*g* for 10 min at 15 °C. The supernatant was aseptically discarded and the cells were brought into the anaerobic chamber and allowed to equilibrate for 5 min to facilitate air exchange in the tube's head space. Anaerobic HGC medium (12 mL) was then added to the tube and the cells were gently resuspended through repeated aspiration.

The microalgal cells suspended in anaerobic HGC (1 mL) was added to the 4 mL HGC medium with experimental Rose Bengal concentrations and the culture tubes were screwed shut, and sealed by wrapping Parafilm®. The tubes were then taken out of the anaerobic chamber and incubated on a roller-mixer for 72 h under 14 h light cycles with ambient temperature conditions (21 °C to 25 °C).

The samples were then centrifuged at 3000 ×*g* for 10 min at 15 °C and the tubes were taken into the anaerobic chamber. There, the supernatant was discarded and the cells were gently resuspended in anaerobic 3 N + BBM (5 mL each) through repeated aspiration. The tubes were sealed once again, taken out of the anaerobic chamber, and incubated on a roller-mixer for 48 h under 14 h: 10 h light cycle with ambient temperature conditions (21 °C to 25 °C).

2.4. Oxic treatment conditions

Oxic treatment of the microalgae was identical with the anoxic treatment noted above albeit without the use of the anaerobic chamber. All sterile transfers and inoculations were carried out under the laminar hood.

2.5. Antibiotic treatment conditions

Antibiotic treatment was carried out as a comparator / control condition. The method used was described previously by Han et al [17], with a few modifications. Briefly, a mixture of Ampicillin, Gentamycin sulfate, Kanamycin, Neomycin and Streptomycin (600 mg/L each) in 3 N + BBM was prepared and sterile filtered using 0.22 μm filter (Millex GV, PVDF membrane). Microalgal cultures (5 mL) were centrifuged at 2000 ×*g* for 10 min at 15 °C and the supernatant was discarded. The microalgae was resuspended in the antibiotics mix (5 mL) and suspended for three days and then transferred to antibiotics-free BBM for five days.

2.6. Characterisation of contaminants

The contaminating microbes in the mother culture were profiled using 16S ribosomal RNA sequencing. The forward primer Bac27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and universal reverse primer: Univ1392R (5′-GGTTACCTTGTTACGACTT-3′) were used. For PCR, initial denaturation was at 95 °C for 5 min; 35 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and extension (2 min at 72 °C); and a final extension at 72 °C for 7 min [18]. The PCR-amplified products

were purified using a subjected to Qiagen clean-up kit QIAquick PCT Purification (CAT:28106) and sequenced at Eurofins using the Eurofins Genomics Mix2Seq platform with Bac27F and Univ1392R primers. BLAST [19] was used to compare the sequences to the 16S ribosomal RNA sequences database in NCBI GenBank database [20].

2.7. Tests for axenicity and viability

Axenicity was deduced by considering the results from three tests:

2.7.1. LB plating

Sterile petri dishes with 10 mL of Lysogeny Broth Agar (Lennox L Agar, Invitrogen™, USA) were prepared. The dishes were divided into three sections and the culture sample (10 μL) was spread in each section. The plates were incubated at 30 °C for five days to check for any growth. If growth was detected, the colony was picked and observed under light microscopy.

2.7.2. BBM plating

BBM plates with agar (2 % w/v) were prepared and 10 μL of sample was spread on the plate and incubated in ambient conditions for five days to check for any growth. If growth was detected, the colony was picked and observed under light microscopy.

2.7.3. Flow cytometry

2.7.3.1. Samples. Samples (1 mL) from the mother culture (0 h) and post treatment culture growth (120*h*) were analysed using a CytoFLEX LX flow cytometer (Beckman Coulter, USA) to compare difference in bacterial contamination. From each 1 mL sample, two 250 μL aliquots were drawn. One aliquot was for staining and the other was the unstained control.

2.7.3.2. Cell fixing. Each aliquot (250 μL) was added to paraformaldehyde (4 % *v*/v, 250 μL) and mixed for 10 s in a vortex mixer, and allowed to rest 10 min at 4 °C. The sample was centrifuged at 3000 ×*g* for 5 min at 4 °C and part of the supernatant (450 μL) was discarded and replaced with 100 μL of paraformaldehyde and mixed again for 5 s using a vortex mixer.

2.7.3.3. Cell staining. To each fixed aliquot (250 μL), SYBR Green I (28 μL, 1:1000 dilution in dimethyl sulphoxide) was added and vortexed for 10 s and allowed to rest in the dark for 15 min. SYBR Green I was obtained from Invitrogen (Thermo Fisher Scientific, USA).

2.7.3.4. Measurement. Autofluorescence was observed using Cytoflex LX and under FSC-A vs R660-APC for chlorophyll and non-chlorophyll pigments. Likewise, FSC-A vs B525-FITC-A was observed for fluorescence from SYBR Green that stained the bacterial DNA, that was distinguished from microalgal cells by size and autofluorescence.

2.8. Final axenic culture

Final purification step was performed using 50 μL samples under the 3 μM anoxic Rose Bengal concentration on BBM agar using the standard four quadrant streak-plating method [21] and incubated for 120 h. No antibiotics were included in the medium. Single colonies from the final quadrant were picked and cultured in 3 N + BBM under the conditions mentioned in Section 2.3. After 36 h of culturing, samples (1 mL) were tested for 16S rRNA amplification and streaked on LB plates to check for bacterial growth. Microscope images of the final culture were taken and compared to the mother culture.

2.9. Statistical analyses

All statistical analyses were preformed using R (4.4.0) [22] with RStudio as the frontend (RStudio 2024.04.1 + 748). Data wrangling and analyses were carried out using the tidyverse set of packages [23]. Flowcytometry data was collected using the proprietary CytExpert software (Version 2.6, Beckman Coulter, USA) bundled along with the CytoFLEX LX (Beckman Coulter, USA), but subsequent analyses was performed using FCS Express software (Version 6.06, Dotmatics, USA).

3. Results

3.1. Quantitative assessment of axenicity

The percent proportions of microalgae and bacterial cells in the culture were quantified using flow cytometry, and the values are provided in Table 1.

In all cases, cell percentage fractions of microalgae and bacteria were lower in anoxic conditions compared the ambient oxic conditions.

3.1.1. Analyses of *N. limnetica* culture

3.1.1.1. Oxic conditions. A one-way ANOVA across the experimental conditions for the microalgae *N. limnetica* in oxic conditions, showed significant difference across the groups ($F_{(4,10)} = 374$, $p = 7.7 \times 10^{-11}$), and a pairwise analysis using Tukey HSD found significant difference in the microalgal population values across all the experimental conditions. For the contaminants, there was significant difference across the groups as well ($F_{(4,10)} = 8.91$, $p = 2.48 \times 10^{-3}$) although a closer inspection using Tukey HSD found that only Antibiotics v/s RB 0 ($p = 0.004$), Antibiotics v/s RB 1 ($p = 0.012$), and Antibiotics v/s RB 3 ($p = 0.003$) showed significant difference in population numbers. This suggests that under oxic conditions, the microalgal population undergo change when exposed to varying Rose Bengal concentrations, while the bacterial concentrations only appear to be statistically different between the control antibiotics treatment and the Rose Bengal concentrations, but not within the experimental Rose Bengal concentrations.

3.1.1.2. Anoxic conditions. The ANOVA across the experimental anoxic conditions found an overall significant difference in the *N. limnetica* population ($F_{(4,10)} = 67.47$, $p = 3.39 \times 10^{-7}$), and pairwise Tukey comparison found similarities between RB 9 v/s RB 3 ($p = 0.12$), RB 3 v/s RB 1 ($p = 0.60$), although RB 9 v/s RB 1 was statistically different at 95 % confidence ($p = 0.013$). When considering changes in the bacterial populations, ANOVA was significant overall ($F_{(4,10)} = 26.39$, $p = 2.7 \times 10^{-5}$), and Tukey HSD found similar trends to the oxic conditions where where no significant difference was found between RB 9 v/s RB 3 v/s RB 1 v/s RB 0 ($p > 0.9$), but the significance was largely driven by the control antibiotic condition versus the Rose Bengal conditions ($p < 0.05$).

3.1.2. Analyses of *D. armatus* culture

3.1.2.1. Oxic conditions. A one-way ANOVA across the experimental conditions for *D. armatus* in oxic conditions, showed no significant difference across the groups ($F_{(4,10)} = 2.774$, $p = 0.87$). This suggests that the chosen Rose Bengal concentrations do not appear to have an effect on the microalgal populations. In the case of bacterial population, there was some statistical significance noted ($F_{(4,10)} = 3.726$, $p = 0.042$), but further Tukey HSD only found marginal statistical difference between Antibiotics v/s RB 1 ($p = 0.045$). This suggests that across the treatment conditions under oxic conditions, very little change could be elicited in the *D. armatus* and its contaminant bacterial population, either through Rose Bengal, or antibiotics.

Table 1
Quantitative presence of microalgae and other microbes in culture using flow cytometry.

RB (μM)	Oxy	*N. limnetica*		*D. armatus*		*C. vulgaris*		*T. obliquus*	
		Algae	Contm	Algae	Contm	Algae	Contm	Algae	Contm
9	Oxic	76.9 ± 4.05	29.82 ± 1.33	3.40 ± 0.54	88.06 ± 1.39	25.95 ± 0.55	5.49 ± 0.20	15.75 ± 0.44	75.33 ± 1.54
	Ano	2.24 ± 0.55	0.16 ± 0.01	0.05 ± 0.04	0.17 ± 0.00	1.64 ± 0.88	0.37 ± 0.06	5.78 ± 0.99	0.65 ± 0.20
3	Oxic	52.74 ± 0.63	45.00 ± 0.44	5.47 ± 0.95	89.78 ± 0.40	20.26 ± 0.02	3.51 ± 0.00	23.74 ± 0.06	85.42 ± 2.56
	Ano	4.09 ± 0.88	0.40 ± 0.47	0.12 ± 0.00	0.01 ± 0.00	5.49 ± 0.06	0.57 ± 0.89	17.71 ± 0.72	0.34 ± 0.08
1	Oxic	59.9 ± 1.30	38.66 ± 0.77	4.30 ± 0.46	90.91 ± 4.12	18.56 ± 5.09	22.60 ± 0.17	24.63 ± 1.11	84.72 ± 1.60
	Ano	5.10 ± 0.13	0.69 ± 0.16	0.06 ± 0.00	0.33 ± 0.03	7.18 ± 1.00	0.83 ± 0.22	12.58 ± 3.65	1.88 ± 0.99
0	Oxic	42.81 ± 1.41	43.81 ± 18.3	6.39 ± 4.19	90.27 ± 5.09	12.95 ± 6.03	55.55 ± 3.61	14.76 ± 5.93	79.66 ± 0.38
	Ano	7.66 ± 0.18	0.66 ± 0.03	0.04 ± 0.00	0.23 ± 0.2	5.87 ± 0.05	1.07 ± 0.12	4.33 ± 0.09	0.67 ± 0.20
Antibiotics		12.50 ± 1.55	10.10 ± 3.21	8.22 ± 0.0	82.34 ± 1.95	25.15 ± 6.00	5.09 ± 1.06	6.98 ± 1.09	51.99 ± 2.31

Values expressed in % total counts recorded.
Oxy = Oxygen conditions of oxic (normal oxygen) or anox (low oxygen).
RB = Rose Bengal,
Contm = contaminating microbes in the culture.

3.1.2.2. Anoxic conditions. ANOVA analysis of the *D. armatus* population under anoxic condition found an overall difference ($F_{(4,10)}$ = 120,710, $p < 2 \times 10^{-16}$) and pairwise Tukey HSD found significant difference between RB 9 v/s RB 3 ($p = 0.006$), RB 3 v/s RB 1 ($p = 0.016$), RB 3 v/s RB 0 ($p = 0.002$), and between Antibiotics and the Rose Bengal conditions ($p < 0.001$). Similarly for the bacterial population, ANOVA found an overall significant difference in the populations ($F_{(4,10)} = 5266$, $p = 2 \times 10^{-16}$), and the Tukey HSD revealed that this was due the differences between the Antibiotics treatment and Rose Bengal concentrations ($p < 0.001$), but not within the experimental Rose Bengal concentrations ($p > 0.9$).

3.1.3. Analyses of *C. vulgaris* culture

3.1.3.1. Oxic conditions. A one-way ANOVA of *C. vulgaris* population across the experimental conditions was found to be significant overall ($F_{(4,10)} = 4.32$, $p = 0.028$), and pairwise HSD found significant difference between RB 9 v/s RB 0 ($p = 0.03$) and RB 0 v/s Antibiotics ($p = 0.043$). In the case of contaminating bacteria, ANOVA found significant difference ($F_{(4,10)} = 517.3$, $p = 1.54 \times 10^{-11}$), and Tukey HSD post hoc found significant pairwise difference across all conditions except RB 9 v/s RB 3, RB 3 v/s Antibiotics, and RB 0 v/s Antibiotics ($p > 0.5$). This suggests that bacterial content changes significantly, but become comparable with antibiotics use at higher Rose Bengal concentrations.

3.1.3.2. Anoxic conditions. Under anoxic conditions, ANOVA found significant difference across the groups ($F_{(4,10)} = 34.94$, $p = 7.52 \times 10^{-6}$) and a Tukey HSD found that the significance was driven largely by the difference between Antibiotics v/s Rose Bengal conditions ($p < 0.001$). This trend was mirrored for bacterial contaminating cells as well with the overall ANOVA being significant ($F_{(4,10)} = 29.57$, $p = 1.62 \times 10^{-5}$) and Tukey HSD revealing difference between Antibiotics and Rose Bengal conditions ($p < 0.001$). These results suggest that under anoxic conditions, differences between the antibiotics treatment and Rose Bengal treatments are significantly different, but the changes within the experimental Rose Bengal concentrations is minimal.

3.1.4. Analyses of *T. obliquus* culture

3.1.4.1. Oxic conditions. Under oxic conditions, *T. obliquus* showed an overall significant difference in the microalgal populations across the test groups ($F_{(4,10)} = 20.89$, $p = 7.62 \times 10^{-5}$) with Tukey HSD revealing significant differences across all the pairwise comparisons except RB 9 v/s RB 0 ($p = 0.99$), and RB 3 v/s RB 1 ($p = 0.99$). In the case of bacterial contaminants, the trends mirrored the microalgal populations, with ANOVA finding significant overall differences across the groups ($F_{(4,10)} = 166.6$, $p = 4.19 \times 10^{-9}$). All conditions showing significantly different bacterial populations except RB 3 v/s RB 1 ($p = 0.99$), and RB 9 v/s RB 0 ($p = 0.09$). This suggests that under oxic conditions, the effect of Rose Bengal at RB 1 and RB 3 are similar and show the highest microalgal population.

3.1.4.2. Anoxic conditions. A one-way ANOVA of the microalgal populations under anoxic conditions found significant overall difference ($F_{(4,10)} = 29.01$, $p = 1.76 \times 10^{-5}$) with Tukey HSD revealing similar microalgal populations in conditions of RB 9 v/s RB 0 ($p = 0.853$), Antibiotics v/s RB 0 ($p = 0.417$), and Antibiotics v/s RB 9 ($p = 0.917$). For bacterial populations, while the ANOVA found significant overall differences between the conditions ($F_{(4,10)} = 1226$, $p = 2.09 \times 10^{-13}$), the significance was largely driven by the difference between the antibiotics treatment and the Rose Bengal concentrations ($p < 0.001$), but not between the experimental Rose Bengal concentrations ($p > 0.5$). This suggests that at concentrations of 1 μM and 3 μM Rose Bengal, microalgal populations undergo change, but the overall bacterial population numbers remain statistically unchanged unless treated with antibiotics.

Based on these values, anoxic conditions were able to decrease the contaminant numbers, and its effect was somewhat amplified by the Rose Bengal depending on the experimental species. To help visualise the performance of the treatments and decide on a suitable condition, a ratio of contaminant bacteria versus microalgae was calculated and plotted, as shown in Fig. 1. Values lower than 1 indicated a lower load of contaminant bacteria relative to the microalgae (i.e. a cleaner culture).

Based on Fig. 1, favourable bacteria to microalgae ratios were obtained at RB 3 μM for *T. obliquus* and *D. armatus*, while the response ratio was relatively indistinguishable between the Rose Bengal concentrations for *C. vulgaris* and *N. limnetica*. However, when considering the percentage microalgal values from Table 1, anoxic conditions with Rose Bengal concentration of 3 μM appears to be universally effective at maintaining sufficient microalgal population while having a relatively low bacterial content.

3.2. Qualitative assessments of axenicity and viability

While the cell numbers in Table 1 showed percentage proportions of cell population, it does not attest to their viability, which was checked with BBM and LB agar plating as shown in Table 2 below.

The BBM plating was performed to check the growth of the microalgae and the LB agar was used to check bacterial growth. However, it was found that *D. armatus* and *T. obliquus* were incapable of growing on LB agar, while *C. vulgaris* and *N. limnetica* were able to grow in these heterotrophic conditions. Distinction between the colonies however was trivial owing to the exclusive presence of chlorophyll in microalgal cells.

3.2.1. Plating profile of *N. limnetica*

In the BBM plating, it was found that *N. limnetica* growth was the

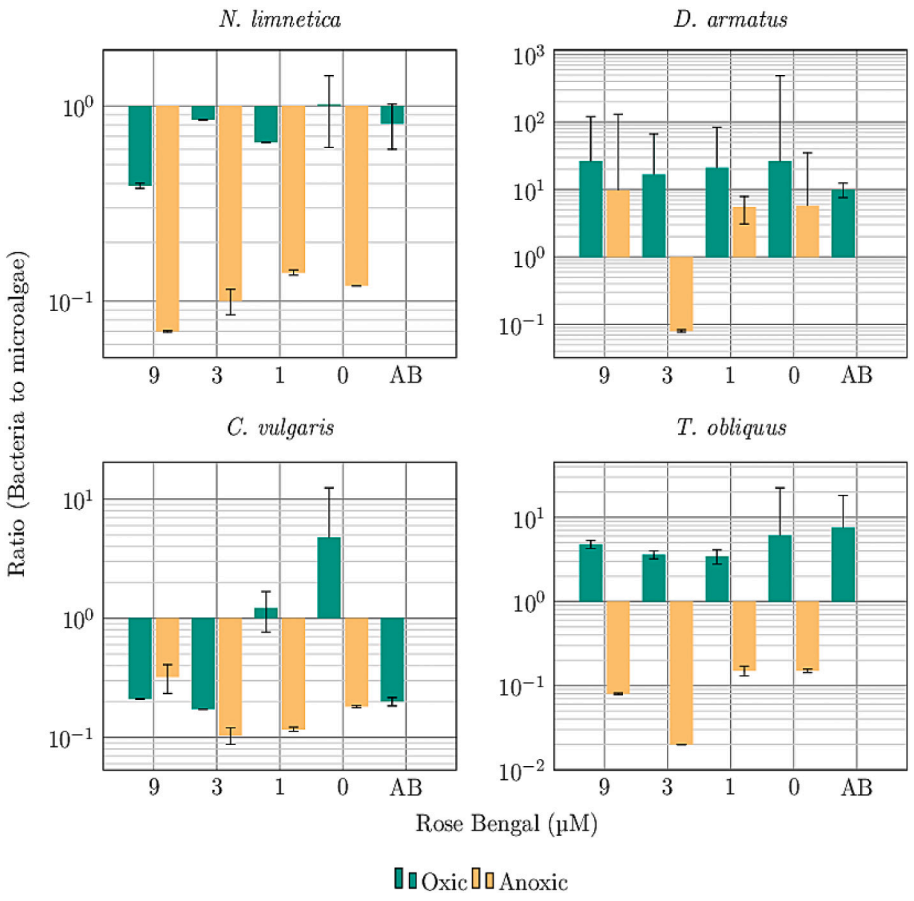

Fig. 1. Ratio of contaminant microbes to microalgae in each treatment condition. Lower the value, the higher the number of microalgal cells relative to the contaminant microbes.

Table 2
Qualitative assessment of the viability and growth of microalgae and bacteria after experimental conditions.

RB (μM)	Oxy	Medium	*N. limnetica*		*D. armatus*		*C. vulgaris*		*T. obliquus*	
			Algae	Contm	Algae	Contm	Algae	Contm	Algae	Contm
9	Oxic	BBM	++	−	+	−	++	+	++	−
	Anoxic		+	−	+	−	+	−	+	−
	Oxic	LB	+++	+	−	++	+	+++	−	++
	Anoxic		+	−	−	+	++	+	−	−
3	Oxic	BBM	++++	−	+	+	+++	+	+++	−
	Anoxic		++	−	+	−	+	−	+	−
	Oxic	LB	+++	++	−	+	+	++	−	++
	Anoxic		++	−	−	+	+	+	−	−
1	Oxic	BBM	++	+	+	−	+++	+	+++	+
	Anoxic		++	−	+	+	+	+	+	−
	Oxic	LB	+++	+++	−	+++	+	+++	−	++
	Anoxic		++	+	−	+	+	+	−	+
0	Oxic	BBM	+++	+	++	+	++	+	++	++
	Anoxic		+	+	+	+	++	−	+	+
	Oxic	LB	+++	+++	−	+++	++	+++	+	++
	Anoxic		+	+	−	++	+	+	−	+
Antibiotics		BBM	++	+	−	+	++	+	+	++
		LB	+	++	−	+	+	++	−	+++

Conditions with "−" indicates no growth.
Oxy = Oxygen conditions of oxic (normal oxygen) or anox (low oxygen).
RB = Rose Bengal,
Contm = contaminating microbes in the culture.

highest at 3 μM Rose Bengal oxic conditions concentration and was the lowest in Antibiotic treatment, 0 uM and 9 uM anoxic Rose Bengal conditions. Bacterial colonies were only observed 1 μM oxic, 0 μM oxic and Antibiotic conditions. This suggests that across all the experimental conditions, viable *N. limnetica* cells were present in the culture.

In the LB plating, bacteria was absent in 3 μM and 9 μM anoxic Rose Bengal concentrations but was detected in all other conditions.

3.2.2. Plating profile for D. armatus

In the BBM plating, *D. armatus* was unviable after antibiotic treatment and showed relatively higher growth in 0 μM oxic Rose Bengal treatment. Bacterial growth was observed in 0 uM oxic and anoxic Rose Bengal concentration, 1 uM anoxic Rose Bengal concentration, and 3 uM oxic Rose Bengal concentration.

In the LB plating, all conditions showed bacterial colonies although they were the fewest in 9 μM and 3 μM anoxic Rose Bengal conditions.

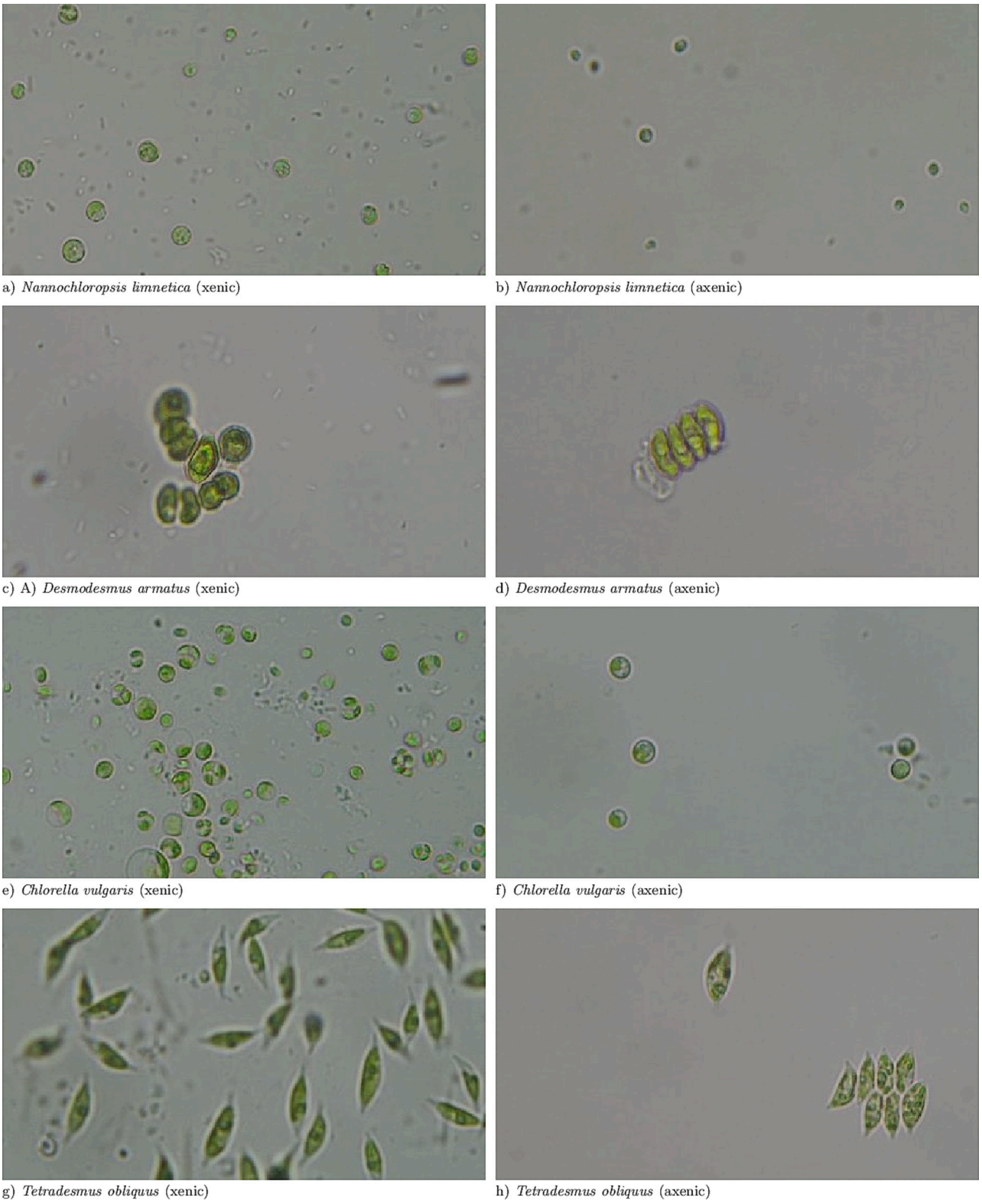

Fig. 2. Comparative images of the microalgal cultures before (A, C, E, and G) and after anoxic 3 μM Rose Bengal treatment (B, D, F, and H) of *Nannochloropsis limnetica, Desmodesmus armatus, Chlorella vulgaris,* and *Tetradesmus obliquus* respectively. Images taken at 40× magnification. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2.3. Plating profile for C. vulgaris

In BBM plating, *C. vulgaris* was able to grow well at Rose Bengal concentrations of 1 μM, and 3 μM in oxic conditions. Bacterial colonies were observed in all oxic conditions, but were absent in all anoxic conditions except at 1 μM concentration.

In LB plating, bacteria showed higher growth in oxic conditions compared to anoxic conditions.

3.2.4. Plating profile of T. obliquus

In BBM plating, *T. obliquus* showed similar trends to *C. vulgaris* and grew best at Rose Bengal concentrations of 1 μM, and 3 μM in oxic conditions.

Similar to *D. armatus*, *T. obliquus* was unable to grow well in LB conditions and served as a good indicator of exclusive bacterial growth. No bacterial growth was observed in anoxic Rose Bengal concentrations of 3 μM and 9 μM.

3.3. Axenic colonies

Based on the results from Tables 1, 2, streak-plating was performed using cultures treated with anoxic Rose Bengal at 3 μM. Individual colonies were picked to obtain cells that were subsequently inoculated in BBM to obtain axenic cultures. These cultures were then subjected to 16S sequencing using the universal primers, but no amplification was observed. Moreover, subsequent LB plating did not find any bacterial colonies (data not shown). Finally, the cultures were manually inspected under a light microscope. Photographs of the microscope images of the culture prior to, and after treatment is shown in Fig. 2 below.

In Fig. 2 A, C, E, and G, xenic mother cultures of *Nannochloropsis limnetica*, *Desmodesmus armatus*, *Chlorella vulgaris*, and *Tetradesmus obliquus* respectively show heavy background of contaminant bacteria while the post treatment samples (B, D, F, and H) show a cleaner profile without any visible bacterial cells.

3.4. Mother culture contaminants

The following contaminants were identified in the mother cultures:

4. Discussion

Photosensitisation has previously been demonstrated to be a good method for targeting metastasised carcinoma, as well as infections that are resistant to traditional antibiotics or chemical treatments. Cossu et al. [9] demonstrated significant decrease in bacterial loads in wash waters with the use of the Rose Bengal dye. The mechanism associated to the observed antibacterial and antiviral effects upon exposure to light using Rose Bengal is attributed to the production of oxygen singlet radicals that result in non-specific cell-membrane damage. The phototrophic nature of the chosen microalgal species allow them to survive for extended periods in anoxic conditions so long as they have adequate access to light for photosynthesis, while the obligate aerobic bacteria were expected to be affected by the lack of oxygen. Based on the results from Tables 1 and 2 however, it was evident that anoxic conditions; though detrimental to bacteria, also affected the microalgal population.

The use of Rose Bengal was thus investigated for its anti-bacterial effects. Based on the bacterial populations in oxic conditions noted in Tables 1 and 2, it was clear that as a standalone treatment, Rose Bengal was ineffective in removing bacterial contaminants within the chosen concentration range. However, in conjunction with anoxic conditions, it was able to synergistically remove bacterial contaminants without significantly affecting the microalgal population. The trend noted in Fig. 1 indicates that ratio of bacteria to microalgae <1 was favourable as the bacterial population was lower than that of the microalgae. In all the species, anoxic conditions with Rose Bengal concentrations of 3 μM was found to produce ratios lower than 1. Gram negative species have negatively charged extracellular membranes, making them particularly resistant to Rose Bengal [24] which is anionic compared to Gram positive bacteria that are known to be susceptible to the compound even under dark conditions [25].

On the contrary, none of the bacterial species noted in Table 3 have evolved to be facultative anaerobes and consequently do not have the ARC (Anoxic Redox Control) to cope with the change in oxic conditions. Dysfunctional ArcA mutants have been known to be more susceptible to sodium dodecyl sulphate (SDS); a negatively charged detergent despite the membrane surface retaining its negative charge [26]. Moreover, divalent ions such as Ca^{2+} and Mg^{2+} are known to promote Rose Bengal uptake in bacteria irrespective of Gram-staining status [27]. This could explain the synergistic effect of Rose Bengal under anoxic conditions despite all the contaminant bacteria being Gram negative.

Among the contaminating microbes identified in the microalgal mother cultures, *Stenotrophomonas* sp. contaminant was identified in the *D. armatus, T. obliquus*, and *N. limnetica* cultures, likely *Stenotrophomonas maltophilia*; a multi-drug resistant, gram-negative, opportunistic pathogen [28]. Similarly a member of the *Sphingopyxis* sp.; likely, *Sphingopyxis chilensis* was also noted in the *D. armatus* and *T. obliquus* cultures. Members of this genus are capable of degrading chlorophenols and have been proposed for bio-remediation. While they are naturally found along natural water-bodies, they are particularly abundant in soils and stagnant water contaminated with pesticides, fertilisers, and other industrial compounds [29]. *S. chilensis* in particular is reported to produce polyhydroxyalkanoate (bio-plastics) [30].

A *Microbacterium* sp. was found to be contaminating the *Chlorella vulgaris* culture with the likely species identified as *Microbacterium foliorum*, which is a common contaminant in most laboratory specimens [31], while *Pseudomonas tianjinensis* is cosmopolitan in distribution and can be found in soils, natural waters, as well as fertilised agricultural lands [32]. Lastly, *Nannochloropsis limnetica* was contaminated by an *Agrobacterium* sp., with the best match being *Agrobacterium radiobacter* which is also commonly found in natural habitats; particularly around farmlands [33]. Depending on the strain, it could be pathogenic to plants. Here we provide a confident genus level identification of contaminants however we cannot be certain of the exact species identified through 16S sequencing alone. Identification of the bacteria at the species level would require deeper sequencing techniques such as using other gene markers or whole genome sequencing.

Lastly, though previous reports indicate antibiotic resistance for *S. maltophilia*, *M. foliorum* and *S. chilensis*, and the lack of decrease in bacterial counts from Tables 1, 2, and Fig. 1 attest to the ineffectiveness of antimicrobial treatment, against these contaminants, further rigorous confirmation would require an antibiogram profiling [34].

Table 3
Bacterial contaminants identified in the microalgal mother cultures using 16S sequencing.

Culture	Genus Identified	Top NCBI species match	Percent Identity
Desmodesmus armatus	*Stenotrophomonas*	*Stenotrophomonas maltophilia*	99.67 %
	Sphingopyxis	*Sphingopyxis chilensis*	97.88 %
Tetradesmus obliquus	*Stenotrophomonas*	*Stenotrophomonas maltophilia*	98.17 %
	Sphingopyxis	*Sphingopyxis chilensis*	98.69 %
Chlorella vulgaris	*Microbacterium*	*Microbacterium foliorum*	98.30 %
	Pseudomonas	*Pseudomonas tianjinensis*	99.53 %
Nannochloropsis limnetica	*Stenotrophomonas*	*Stenotrophomonas maltophilia*	99.44 %
	Agrobacterium	*Agrobacterium radiobacter*	99.50 %

All identified bacteria were gram-negative species, with *S. maltophilia, M. foliorum* and *S. chilensis* known to be multi-drug resistant.

Since no single method can unequivocally attest to axenicity [35], the status was investigated by considering three pieces of evidence gathered from light microscopy, LB plate growth, and 16S PCR. Other methods such as confocal microscopy [36], 16/18 s amplification [37], and electron microscopy [35,37] have been reported by other researchers.

4.1. Caveats and considerations

There are significant considerations when applying anoxic Rose Bengal to achieve axenic cultures; many aspects that could not be addressed within the experimental setup described herein. Firstly, all the contaminating microbes identified (Table 3) were obligate aerobes. Thus, the anoxic conditions were able to significantly affect their populations within the mother culture. Moreover, the cellular uptake required for the synergistic antimicrobial action of Rose Bengal despite the bacteria being gram-positive, was made possible only by the uptake enhanced by the Ca^{2+} and Mg^{2+} ions that are generally available in higher concentrations in BBM compared to natural habitats to promote microalgal growth. The effectiveness of the described treatment is unclear if facultatively anaerobic microbes were to contaminate cultures under low Ca^{2+} and Mg^{2+} concentrations.

Secondly, only bacterial contamination was addressed in this work as fungal, amoebic, or other contaminating lifeforms were not encountered within the mother cultures and were consequently not considered as part of this investigation. The effectiveness of the anoxic Rose Bengal in the proposed 3 μM concentration remains unclear under such contaminating conditions.

Lastly, this method was designed to only serve in unialgal cultures; i.e., only one microalgal species in a given mother culture. Although different microalgae showed different degrees of viability to the anoxic Rose Bengal treatment (Table 2), and this may be leveraged to obtain pure cultures of the more resistant microalgae, physical methods such as micropicking, microfluidics or flow-cytometry may be a more suitable intermediate step to separate individual microalgal species. Moreover, the efficacy of the proposed method has only been demonstrated with three green algae and one eustigmatophyte and it may be important to demonstrate it further with examples from diatoms, dinoflagellates, etc.

5. Conclusion

Decontamination appears to be largely a function of anoxy that is synergistically enhanced by Rose Bengal at concentrations ≥3 μM. Most cultures were contaminated by Gram negative bacteria, a couple of which were multi-drug resistant and consequently, antibiotic treatment was ineffective in decontaminating the cultures.

CRediT authorship contribution statement

A. Iyer: Writing – original draft, Methodology, Investigation, Funding acquisition, Conceptualization. **M. Monissen:** Writing – review & editing, Validation, Methodology, Investigation. **Q. Ma:** Software, Methodology. **M. Osborne:** Software, Resources, Methodology. **E. Schaedig:** Writing – review & editing, Validation, Resources, Methodology. **O. Modin:** Supervision, Funding acquisition. **R. Halim:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

Ajay Iyer reports financial support was provided by Irish Research Council. Matthias Monissen reports financial support was provided by Erasmus mobility programme. Qinge Ma reports financial support provided by Department of Agriculture, Food and the Marine (Ireland). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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