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Foam flotation can remove and eradicate ciliates contaminating algae culture systems

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Abstract: We demonstrate, for the first time, the efficacy of a surfactant-aided foam flotation system to remove and eradicate ciliates contaminating microalgae cultures. Using sodium dodecyl sulphate (SDS) as the surfactant, ciliates removal efficiencies of up to 86.6% were achieved from pure ciliates cultures at an SDS concentration of 40 mg L⁻¹. At this concentration the majority of ciliates were lysed due to increase in SDS concentration in the collected foamate. The removal efficiency decreased to 55.0 % in mixed algae-ciliates cultures however, this was compensated by employing a multistage flotation and SDS (50 mg L⁻¹) reuse strategy that achieved removal efficiencies of 96.3%, lysing all collected ciliates, but not affecting microalgae growth. The chemicals cost for the process was US\$ 0.0025 per m³ substantially less than comparator treatments. Building upon its applications in biomass dewatering, pre-processing and sterilising, we add metazoan contamination control to the utilitarian properties of foam flotation for the microalgae biotechnology sector.

Keywords: biofuel; microalgae; harvesting; grazers; open ponds; raceways

Introduction: The industrial scale production of microalgae typically occurs in open culture systems such as ponds and raceways and in closed photobioreactor systems. Photobioreactors afford the grower much greater control over the culture environment, whereas open culture systems are, to all intents and purposes, a microcosm of natural waterways; incorporating the risks posed to microalgae from pathogens and grazers. In natural systems microalgae blooms are kept in check by an assortment of biotic actors, including; viruses [1], bacteria [2], parasites [3], protists (including ciliates) [4, 5], zooplankton [6], and fungi [7]. However, such biological

control can be catastrophic for algae culture systems, particularly when attempting to culture at industrial scale.

Reflecting the equivalence to natural systems, the type of contaminants found in ponds and photobioreactors (the ubiquitous bacteria and viruses aside) include; parasites [8], protists [9], fungi [10], amoebae [11], and rotifers [12]. There is therefore an imperative to develop cost-effective, robust and scalable culture management strategies that either prevent contamination, severely limit the population growth of the contaminant, and/or can safely remove the contaminating agent without harming the microalgae or necessitating major disruption to culture operations. This desire for contaminant control has vexed mass algae growers for over half a century [13-15] and the microalgae industry continues to wrestle with contaminant management [16-18], spawning growing research fields in monitoring and modelling of contamination scenarios [19-24].

Current approaches to culture management include growing species or strains that tolerate abiotic factors (e.g. temperature and salinity) out with the tolerance range of their predators or competitors; *Dunaliella salina* culture being a prime example [25]. Rather more interventionist methods include dosing with chemicals such as pesticides [26-28] and disinfectants [29, 30]; however, there remains the risk of causing inadvertent harm to the algae [31], or worse, to the culture personnel. Alternative ecological-based control strategies have been investigated [32-35].

Foam flotation, a technique to separate solution components (including microalgae [36]) by exploiting the variation in their surface charges, is a hitherto unexplored technology for contaminant removal. The closest approximation is that of Kamaroddin et al. [37] who used a microbubble-driven airlift loop bioreactor, switching between CO₂ and ozone as carrier gases, to demonstrate that it was possible to both disinfect and harvest *D. salina* cultures. Building upon work in surfactant-aided foam flotation which demonstrated the efficacy and economic merits of the approach for dewatering and pre-processing microalgae biomass [38-40], the use of foam flotation for the removal of contaminating ciliate from cultures of *Chlorella vulgaris* was investigated.

2. Materials and methods

2.1 Microalgae and ciliates culture

Chlorella vulgaris (CCAP 211/63) was cultured in BG-11 medium in 10 L polycarbonate carboys (Nalgene) at 18 ± 2 °C and a 16L: 8D photoperiod with a mean luminance of 2,500 Lux. The cultures were continuously aerated using an aquarium pump (Blagdon KOI AIR 50).

The microalgae were grown for three weeks, reaching a cell density of $3.28 \times 10^7 \pm 10^6$ cells mL⁻¹.

The ciliate *Tetrahymena pyriformis* (CCAP 1630/1W) was purchased from the Culture Collection of Algae and Protozoa, UK. *T. pyriformis*, despite not being a microalgae grazer, was chosen for this lab test as it is a well-researched organism that may be considered as a model to begin evaluating and optimizing our foam column separation technique, which can subsequently be extended to other protozoa species that pose a threat to microalgae cultivation. One of the benefits of using *T. pyriformis* is its rapid growth rate, with a population doubling time of 5-7 hours under optimal conditions [41]. The culture was grown axenically at 18 ± 2 °C, pH 6.5 – 7.5 in autoclaved proteose peptone yeast extract medium (PPY) containing 2% proteose peptone and 0.25% yeast extract (Sigma Aldrich, UK). The medium was stored at 4 °C and subsequently adjusted to 15 °C prior to use. A dense *T. pyriformis* culture was gently agitated to evenly mix the ciliates and 0.5 mL was transferred to 10 mL test tubes containing 5 mL of PPY medium using a sterile Pasture pipette. After seven days the density of each new culture was checked using an LEICA DM500 inverted microscope at $\times 200$ magnification. The test tube junction cap was subsequently wrapped with white plastic nylon to reduce evaporation.

2.2 Foam flotation column

The foam flotation column was a modified version of that described by Coward et al. [38] (Fig. 1); briefly, a sparger made from 6.0 mm thickness fine grade polyethylene sheet was used to provide bubbles with an average diameter of 1.13 ± 0.14 mm. The column section was made from a series of modular sections of 250 mm in length, 47.5 mm internal and 51.5 mm external diameter. The column height was 510 mm. A collection cup was attached to the top of the column to receive the foam. The total column volume was approximately 1.0 L. The foam harvester was designed as a foam separation column for the physical separation of the ciliates.

2.3 Ciliates multistage harvesting using the foam column

Three sodium dodecyl sulphate (SDS) concentrations (20, 30 and 40 mg L⁻¹; Sigma Aldrich, UK) were made in reverse osmosis water and tested in a foam column operating in batch mode. Two experiments were conducted at airflow rates of 1.0 and 2.0 L min⁻¹. Air was fed to the sparger to produce bubbles which led to the formation of a surfactant stabilised foam. The foam flowed up the column and was collected in a collection cup. Before each trial, 50 mL of *T. pyriformis* culture was added to 950 mL of SDS solution. To ensure sufficient space within the column for foam to form, each run consumed 250 mL of the SDS-ciliates solution; allowing

for four replicate runs per condition. The foam collected from each experiment was allowed to collapse, was collated and the volume determined. The *T. pyriformis* cell density in the foam and the remaining liquid phase was measured using an improved Neubauer haemocytometer and an inverted light microscope. A drop of Lugol solution was used to immobilise the ciliates for counting. Each column run lasted for a maximum duration of 30 min or until it was no longer possible to collect any more foam, whichever came first. The collated foam (ca. 10 mL) with an assumed high concentration of recovered SDS (including ciliates) was returned to the culture liquors and a further run was conducted as described above. In total, three runs were conducted per SDS concentration as part of a multistage ciliates harvest (the fourth run did not produce any further foam). The aforementioned experiments were repeated but with the addition of *C. vulgaris* ($3.28 \times 10^7 \pm 10^6$ cells mL⁻¹). To control for any effect of air pressure on the ciliates, 100 mL of *T. pyriformis* culture was transferred into the foam column and subjected to 2 L min⁻¹ air flow without any SDS for 30 min. The numbers of ciliates in the foamate and the Removal Efficiency (RE) were determined using equations 1 and 2 respectively.

$$\text{Ciliates in foamate} = A - B \text{ cells mL}^{-1} \text{ (Eqn 1)}$$

Where: *A* is the initial ciliates count prior to each run, and *B* is the final ciliates count after each run.

$$RE = \frac{\text{Ciliates in foamate}}{\text{Initial ciliates count}} \times 100 \text{ (Eqn 2)}$$

2.4 Determining the effect of SDS on the ciliates

Initial observations during harvesting suggested that the highest SDS concentration caused the ciliates to lyse; therefore an experiment was conducted to determine the lowest SDS concentration beyond which the ciliates could not survive. Four SDS concentrations (40, 44, 48 and 52 mg L⁻¹) were prepared and added to 1 L of algae/ciliates culture. The ciliates were monitored on an hourly basis using an inverted microscope and a haemocytometer.

2.5 Multistage foam column ciliates eradication using high SDS concentration.

Twenty millilitres of *T. pyriformis* (ca. $10,000 \pm 10^3$ cells mL⁻¹) was added to 980 mL of *C. vulgaris* and mixed with 50 mg SDS. The mixture was allowed to stand for two hours. A control sample was also prepared without SDS. The concentrations of both *C. vulgaris* and *T.*

pyriformis prior to, and after the experiment were determined. The foam column described previously was used to recover the SDS (ca. 10 mL) from the culture. The recovered SDS was then added to fresh algae/ciliates culture without SDS making the whole volume to one litre. The mixture was allowed to stand for 12 hours before conducting another foam column experiment to recover the used SDS. The further two runs were conducted (each allowed to stand for 24 hours), thereby treating four litres of algae/ciliates culture with an initial SDS concentration of 50 mg with the total time taken to complete the experiment being 62 hours. The variation in timings from 2 to 24 hours before recovering the SDS for the 4 stages was to compensate for the assumed slight decrease in the SDS concentration within the collated foamate.

2.6 Data Analysis

Data presented are the mean values of three separate experiments, with each experiment comprising four (250 mL) replicates. Statistical analyses were done using the Statistical Package for the Social Sciences (SPSS) software analysis and Minitab v17. Data were tested for normality using a Shapiro-Wilk's Test and for equality of variance using a Levene's Test. Normally distributed data were compared using an Analysis of Variance (ANOVA) test. Data that were not normally distributed were analysed using the Levene's test. A repeated measures analysis of variance (rANOVA) was used to analyse the interaction of the three cycles of SDS reuse and between the SDS concentration and air flow rate on the ciliates removal efficiency. A General Linear Model was used to analyse ciliates regrowth with time and SDS concentration.

3. Results and Discussion

At industrial scales of microalgae production, contaminants such as ciliates are introduced via the air, the culture media and vessel, and the starter culture [42], with heavy ciliates infection in outdoor microalgae cultures reported by Moreno-Garrido and Canavate [29]. Neutralising or removing these predator ciliates may enhance the growth rate, health and robustness of algae cultures without substantial disruption to farm operations.

3.1 Harvesting ciliates without microalgae

The addition of 20, 30 and 40 mg L⁻¹ SDS into the *T. pyriformis* culture without *C. vulgaris* showed no immediate physical effect on the ciliates; however, after 60 min cellular debris

began to appear in the 40 mg L⁻¹ treatment. The initial harvesting trials yielded mean removal efficiencies of 56.95, 79 and 86.59% for 20, 30 and 40 mg L⁻¹ SDS respectively at 2 L⁻¹ air flow (Fig. 2A). Post harvesting, it was observed that many of the ciliates had lysed in the SDS treatments. This is likely due to an exponential increase in SDS concentration in the foamate, potentially in the order of a 400% increase [38]. The treatment without SDS (i.e. the air pressure control) resulted in no damaged ciliates.

3.2 Harvesting ciliates with microalgae

Removal efficiency declined relative to the algae-free trials (Fig. 2B and first stage removal in Fig. 3) at all SDS concentrations. The added algal component interjects greater complexity to the suspension, which now involves ciliate–SDS–algae interactions rather than just ciliate–SDS. The cations around the algae could interfere with the SDS–ciliate interactions, reducing the ciliates' contact area and thus decreasing removal efficiency. This was not possible when only SDS solution was used which resulted in a higher removal efficiency. Removal efficiency increased markedly (rANOVA $F = 117.06$, $p < 0.001$) following a multistage removal strategy at all SDS concentrations wherein the collected SDS was returned to the culture followed by two further column runs (Fig. 3). The highest removal efficiency (96.25%) was obtained on the third stage using 40 mg L⁻¹ SDS at a 2 L min⁻¹ airflow rate. To fully eradicate the ciliates from the algae culture and to eliminate recolonization from within the same population, the ciliates RE must be 100%; regrettably this was not achieved using this approach.

It was hypothesized that an anionic surfactant such as SDS could aid the removal of *T. pyriformis* from algae cultures as their cell membrane is usually covered with Ca²⁺ and K⁺ ions [43-45]. Therefore, in theory, the ionic interaction between the anionic SDS and the ciliates should increase the hydrophobicity of the ciliates, thus enhancing their removal potential. SDS was also chosen as it is a relatively poor collector for microalgae [46]; therefore in theory, the ciliates would be removed leaving the microalgae *in situ*. This was found to be the case, and whereas some microalgae were incidentally collected in the foamate the number of cells was very low (RE < 2%).

3.3 Effect of SDS on ciliates

The gradual increase in SDS concentration beyond 40 mg L⁻¹ had a clear lysing effect on the ciliates with time, with both concentration and time being significant factors (concentration: F

= 5.4, $p = 0.002$; time: $F = 3.51$, $p = 0.01$). SDS concentrations from 44 mg L⁻¹ and above completely lysed the ciliates within six hours (Fig. 4). The highest effect was at 52 mg L⁻¹ with lysis within two hours. However, at SDS concentrations of 40 mg L⁻¹ and below, the ciliates population quickly recovered and dominated the culture within 48 – 72 h.

3.4 Multistage ciliates control using high SDS concentration and foam column

Based on this knowledge of the susceptibility of *T. pyriformis* to SDS, a 50 mg L⁻¹ concentration was used to treat four one litre batch algae cultures. Following treatment, the algae cultures grew ciliates free, increasing to 6.28×10^8 from 1.44×10^8 cells mL⁻¹ within seven days. The microalgae were found to be free of cell damage. Ciliates control using a multistage SDS recovery and reuse strategy proved to be faster (15 h 30 min) and offer an economic advantage over other ciliates chemical control measures (Table 1). For instance, Ashraf et al. [47] used 60 g L⁻¹ sodium chloride and 80 mg L⁻¹ quinine to control ciliates in outdoor microalgae cultures over a 24 hour period. Similarly, some of the chemicals used to control ciliates such as sodium chloride and oxytetracycline can also negatively affect the microalgae [47]. In a separate experiment conducted to determine the effect of SDS on *C. vulgaris* it was discovered that up to a 100 mg L⁻¹ exposure had no physical effect on the microalgae. The exposed algae were successfully recultured and normal growth maintained; possibly due to the *C. vulgaris* cell wall being more resistant to SDS than the cell membrane of *T. pyriformis*.

4.0 Summary

Some of the methods of dealing with ciliates contamination include chemical additives such as quinine [29], filtration techniques and manipulating environmental factors such as pH, light and air [48]. However, these approaches have drawbacks in terms of high energy consumption, maintenance cost as well as possible side effects from the chemicals. Our approach to overcome such constraints was to use SDS-aided foam flotation to remove and eradicate the ciliates and subsequently recover the SDS for reuse.

The foam column approach, if optimized and scaled to work with photobioreactors and open pond culture systems (as demonstrated in the minerals extraction industries [49, 50]), could offer advantages for removing biological contaminants with similar characteristic to ciliates, particularly those with fragile cell membranes, whilst retaining the majority of the algae cells in the culture. It would also be important to test the approach on cultures with a much lower

infestation intensity to validate it as a potential routine control measure. The lab scale system demonstrated here, may be particularly useful for laboratory-scale end-users and/or the maintenance of stock cultures, and testing on a range of microalgae with differing cell wall properties should be the next step in development.

Author contributions

This study was designed and conceived by JGML, GSC and AU. Data were collected by AU and analysed by AU and GSC. The manuscript was written by AU and GSC, and edited and approved by JGML. All authors have agreed to the submission of the manuscript.

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Figure legends

Fig. 1. The foam flotation column dimensions: foam collection cup, 300 mm diameter, 100 mm tall; flotation column, 510 mm tall, 51.5 mm outer diameter, 47.5 mm inner diameter; polyethylene sparger, 6.0 mm thickness.

Fig. 2. Removal efficiency of *T. pyriformis* at an air flow rate of 2 L min⁻¹ with different SDS concentrations: A) without the presence of microalgae; and, B) with microalgae. Mean ± St. Dev.

Fig. 3. Removal efficiency of *T. pyriformis* in a mixed algae–ciliates culture with different SDS concentrations as part of a multistage ciliates removal and SDS reuse strategy, at two air flow rates: A) 1 L min⁻¹, and B) 2 L min⁻¹. Mean ± St. Dev.

Fig. 4. Survival and population regrowth of *T. pyriformis* following exposure to a range of SDS concentrations. All ciliates had died within six hours with the exception of the 40 mg /L treatment. Mean ± St. Dev.

Fig. 1

Fig. 2

Fig. 3.

Fig. 4.

Graphical abstract

Table 1: Comparison of chemical methods for controlling ciliates and their associated costs (from [47]). Costs (US\$) were derived from the mean value of the bulk chemical price. Currency rates correct as of 05/04/2017.

Chemical treatment	Dose L ⁻¹	Cost L ⁻¹
Falcon Killer Powder (pyrethroid 0.5 % w/w)	4 g, no effect on either ciliates or algae	
Oxytetracycline (liquid)	3 mL killed both ciliates and algae after 24 h	
Sodium chloride	60 g killed ciliates after 24 h	\$ 0.250
	90 g killed both ciliates and algae after 24 h	
Quinine sulfate	80 g killed ciliates after 3 h while both ciliates and algae died after 24 h	\$ 0.016
SDS (present work)	12.5 mg killed ciliates in 15 h 30 min	\$ 0.0025

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