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Published in:
Algal Research

DOI:
10.1016/j.algal.2019.101570

Publication date:
2019

Document Version
Peer reviewed version

Citation for published version (APA):
Deruyck, B., Nguyen, T. K. H., Ramasamy, P., & Muylaert, K. (2019). Low doses of the quaternary ammonium salt Cetyltrimethylammonium bromide can be used as a pesticide to control grazers in microalgal cultures. *Algal Research*, *41*, [101570]. https://doi.org/10.1016/j.algal.2019.101570

Low doses of the quaternary ammonium salt cetyltrimethylammonium bromide can be used as a pesticide to control grazers in microalgal cultures

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Abstract: Contamination of large-scale microalgal cultures by grazers can cause huge losses in biomass productivity. Here we propose the use of a quaternary ammonium salt CTAB (cetyltrimethylammonium bromide) to eradicate three types of commonly occurring grazers in microalgal cultures: the rotifer *Brachionus*, the ciliate *Sterkiella* and the flagellate *Paraphysomonas*. Low, premicellar doses ($\leq$ 3 µM) of CTAB rapidly eradicated (within 1 – 2 d) all three tested grazers from microalgal cultures without significant losses ($p < 0.05$) in microalgal productivity. However, doses exceeding 5 µM also negatively affected microalgal growth. The optimal dose of CTAB that resulted in complete eradication of the grazers with minimum impact on microalgal productivity was 3 µM for *Brachionus*, 2 µM for *Sterkiella* and 3 µM for *Paraphysomonas*. Thus, being a readily available chemical, CTAB has the potential to be used as a fast-acting, low-cost control agent against a range of frequently occurring grazer types in large-scale microalgal cultures.

Keywords: Microalgae; contamination; ciliates; rotifers; flagellates; CTAB

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1. Introduction

During the past two decades, there has been an increased interest in using microalgae for food, nutraceuticals, feed and biofuel production. Today, microalgae are most commonly produced in open raceway ponds [1]. These large-scale cultivation systems often experience severe losses in productivity due to microscopic grazers that invade the cultures and feed on microalgae, such as rotifers, ciliates and flagellates [2,3]. These grazers form resting stages that are easily dispersed through air. As a result, contamination of microalgal cultures by these grazers is hard to avoid. Open raceway ponds are particularly susceptible to contamination because the water surface is continuously exposed to the atmosphere [4]. Nevertheless, closed systems such as photobioreactors are also susceptible to contamination because they require continuous sparging with large volumes of air to remove excess oxygen [5], and this air may contain dormant stages of grazers. Once entering the culture, the grazers will emerge from the dormant stages and will rapidly develop a large population by feeding on microalgae and can cause a culture crash within few days [6,7].

In recent years, some methods have been proposed to control grazers that have invaded microalgal cultures. Addition of ammonium (NH_4^+) in combination with an increase in pH results in the formation of free ammonia (NH_3), which is toxic to some but not all grazers [8]. CO_2 asphyxiation can be used to control rotifer, ciliate and flagellate contamination [9,10]. Use of chemicals such as quinine sulfate [4], toosendanin [11] and rotenone [12] have also been proposed. Recently, feeding deterrents from marine algae and their chemical analogues have been reported to control the grazers [13]. However, most of these methods either require large doses of chemicals or are not cost-effective be used in large-scale systems. Therefore, it is important to find methods that are both effective at low dose and low-cost, and that are active for controlling grazers in large-scale microalgal cultures.

Quaternary ammonium compounds (QACs) are widely used as antimicrobial agents in detergents and cosmetics [14,15]. CTAB (cetyltrimethylammonium bromide), a widely used QAC, is a cationic surfactant with a hydrophobic chain linked to a positively-charged quaternary ammonium head group. CTAB has been effectively used against bacteria and fungi at varying concentrations: 44 µM to 3 mM [16]. The main cause of QACs toxicity on living cells is due to interaction with the cell membrane [17]. The positively-charged quaternary ammonium head of CTAB interacts with negatively-charged

phospholipids of the plasma membrane, followed by penetration of the hydrophobic tail of CTAB inside the bilayer to interact with the lipidic tails through hydrophobic exclusion. These series of events alter the membrane integrity to result in leakage of intracellular components and ultimately lead to cell death [18].

The aim of this study was to assess if CTAB can be used as a chemical control agent to eradicate grazers from microalgal cultures. To test this, we evaluated the impact of CTAB on three different types of microalgal grazers that are known to invade large-scale microalgal cultures: rotifers, ciliates and flagellates [2,19]. Earlier studies, however, also reported a negative impact of CTAB on microalgae in natural aquatic ecosystems [15]. Therefore, we also assessed the impact of CTAB on the microalgae and aimed to find an optimal dose that results in rapid eradication of grazers with minimal impact on microalgal productivity.

2. Materials and methods

2.1. Cultivation of microalgae

Monocultures of microalgae *Chlorella vulgaris* 211-11 B (SAG, Göttingen) and *Chlamydomonas reinhardtii* 77.81 (SAG, Göttingen) were maintained in 2 L batch cultures in Wright's Cryptophyte (WC) medium [20] in a temperature-controlled room (20 ± 1 ºC) at a light intensity of 80 µE m^{-2} s^{-1} and light-dark cycle of 16:8 h. Growth of microalgae was monitored spectrophotometrically by measuring the absorbance at 750 nm (OD_{750}). Biomass concentrations (mg L^{-1}) were calculated by multiplying the OD_{750} values with 353 for *Chlorella* and with 551.2 for *Chlamydomonas*, derived from the plot of OD_{750} values versus varying dry weight (DW; see SI for details). Late exponential phase cultures were used for the experiments.

2.2. Cultivation of rotifers, ciliates and flagellates

The rotifer *Brachionus calyciflorus* and the flagellate *Paraphysomonas* were isolated from a freshwater pond near the KU Leuven campus in Kortrijk, Belgium (**See SI**). The hypotrich ciliate *Sterkiella* was isolated from a rainwater storage reservoir in Meulebeke, Belgium (**See SI**) [21].

Brachionus were maintained in batch cultures for approximately 3 years before experimentation under the same conditions as used for microalgae. *Brachionus* cultures were routinely transferred every 4 – 5 days to a fresh *Chlorella* culture: 25 mL of the rotifer culture was transferred to a 0.5 L glass bottle

containing 200 mL WC medium and 100 mL of an exponential phase *Chlorella* culture. Initial rotifer density varied from 0.1 to 1 rotifers mL^{-1} and the *Chlorella* concentration varied from 0.1 to 0.35 g L^{-1}. *Sterkiella* and *Paraphysomonas* were maintained in 6-well plates in 3 mL volume cultures for approximately 8 months before experimentation. Both grazers were transferred every 3 days: 0.5 mL of the ciliate/flagellate suspension was transferred to a well containing 2 mL of WC medium and 0.5 mL of an exponential phase *Chlamydomonas/Chlorogonium elongatum* 30.98 (SAG, Göttingen) culture. Initial ciliate/flagellate abundance varied from 10 to 50 cells mL^{-1} and the microalgal food concentration varied from 0.08-0.19 g L^{-1}. To obtain sufficient numbers of *Sterkiella* and *Paraphysomonas* cells for this study, cultivation was upscaled to 350 mL batch cultures with the same transfer conditions. Exponential growing populations of all grazers were used as inocula for all conducted contamination experiments.

2.3. Evaluation of grazer elimination by CTAB

A working solution of 100 µM of cetyltrimethylammonium bromide (CTAB; analytical grade; Sigma-Aldrich, Belgium) was freshly prepared for each experiment by diluting the stock solution (10 mM) in WC medium. Different concentrations of CTAB (0.1 – 9 µM) were tested against the grazers in microalgal cultures and the results were compared against the untreated control (**see SI Table S1**). Further, the effect of CTAB on microalgal monocultures was also tested with the selected concentrations.

Separate controlled contamination experiments were carried out for each grazer. Experimental setup consisted of 100 mL microalgal cultures bubbled with sterile-filtered air and gentle stirring (magnetic stirrer, 10 rpm) in the presence or absence of grazers. Each treatment was tested in triplicate and all treatments were incubated in the similar culture conditions as for the microalgal monocultures. Initial microalgal and grazer levels were chosen as to represent a high-risk level of infection in established microalgal cultures (**See SI Table S2**). Previous experiments showed that these initial concentrations result in culture crash within few days (e.g. as in [21]). For each treatment, CTAB was added ~ 5 minutes after inoculating the microalgal cultures with the grazers. The cultures were monitored daily up to 5 days. Growth of microalgae was monitored spectrophotometrically (OD_{750}) from 2 mL subsamples. The abundance of rotifers and ciliates was determined from 2 mL subsamples using a Sedgewick rafter counting chamber under an Olympus SZX10 stereomicroscope. Rotifer and ciliates counts were derived

from formaldehyde (5% vol)-preserved samples. Abundance of flagellates was determined from 2 mL subsamples under an Olympic BX51 microscope (200X magnification) using a Bürker counting chamber in presence of glycerol (final concentration of 10% w/vol).

2.4. Growth rate

The specific growth rate (μ) of grazers was calculated by fitting their abundance from 24 h to 72 h of cultivation to an exponential function.

$$\mu = \frac{ln\ (N_2/\ N_1)}{t_2 - t_1}$$

Where, μ represented the specific growth rate, N_1 and N_2 are the abundance at times t_1 and t_2.

2.5. LD_{50} determination

The 24-h LD_{50} (the dose lethal to 50% of the individuals in a population over 24 h) of CTAB was determined for microalgae and grazers. For the microalgae, the rotifer *Brachionus* and the ciliate *Sterkiella* LD_{50} values were determined through a linear regression curve. Whereas, for the flagellate *Paraphysomonas*, the mortality as a function of CTAB concentration was best described by a sigmoid curve, which was further linearized through probit analysis method described by Finney (1952) [22] to determine the LD_{50} values (see SI for detailed methodology and regression equations).

2.6. Statistical analysis

Data were statistically analysed using R version 3.4.3 (The R Foundation for Statistical Computing, Austria). The independent and interacting effects of grazers and CTAB on final microalgal biomass concentration were analysed using two-way ANOVA followed by Tukey's HSD test for all pairwise comparisons. The influence of varying CTAB concentrations on final grazer densities were compared using a one-way ANOVA followed by Tukey's HSD test for all pairwise comparisons. Significances for the differences were established at an $\propto$ risk of 5% ($p = 0.05$).

3. Results and discussion

3.1. Impact of CTAB on microalgal grazers and microalgae

To test the efficacy of CTAB as a grazer control agent in microalgal cultures, controlled contamination experiments were performed. Microalgal growth and culture biomass productivity in the

presence or absence of grazers exposed to different CTAB concentrations were compared (growth curves in SI **Fig. S1).** In control cultures without CTAB and grazers, *Chlorella* biomass doubled within 48 h from 160 mg L^{-1} to approximately 320 mg L^{-1}, whereas *Chlamydomona*s biomass doubled within 72 h from 135 mg L^{-1} to approximately 270 mg L^{-1}. When the culture was contaminated with grazers and no CTAB was applied, a negative impact of the grazers on the microalgal biomass production was observed, resulting in a maximum biomass loss within 72 h. The daily mean biomass loss was 32% for *Chlorella* contaminated with *Brachionus*, 26% for *Chlamydomonas* contaminated with *Sterkiella* and 21% for *Chlamydomonas* contaminated with *Paraphysomonas* **(Fig. 2).** At the same time, the abundance of grazers increased exponentially for *Brachionus* from 5 ind mL^{-1} to 175 ind mL^{-1} within 72 h, for *Sterkiella* from 25 cells mL^{-1} to 435 cells mL^{-1} within 96 h and for *Paraphysomonas* from 1,000 cells mL^{-1} to 1.75 x 10^3 cells mL^{-1} within 96 h. Further, their specific growth rate between 24 and 72 h was as high as 1.3 ± 0.08 d^{-1} for *Brachionus*, 0.9 ± 0.03 d^{-1} for *Sterkiella* and 2.11 ± 0.03 d^{-1} for *Paraphysomonas*. This shows the vigour and severity of impact of these grazers on the productivity of microalgal cultures.

Addition of CTAB had a negative impact on all three grazer types and this impact was concentration-dependent **(Fig. 2)**. Significant reduction ($p<0.05$) in abundance of *Brachionus* was observed at doses ≥1 µM, when compared to the untreated control. Whereas, for *Sterkiella* a dose as low as 0.1 µM already resulted in significant reduction ($p<0.05$) in their abundance. In contrast, significant reduction ($p<0.05$) of *Paraphysomonas* occurred only at doses ≥2.5 µM **(Fig. 2)**. Doses higher than 3 µM resulted in complete eradication of all three grazers within 72 h. These results indicate that CTAB could be used to completely eradicate all three grazers from the microalgal culture.

CTAB also negatively impacted the growth of microalgae with doses higher than 4 µM (**Fig. 1**). In fact, addition of CTAB at higher doses had negative impact on both the tested microalgae even in the absence of grazers. The toxicity of CTAB on microalgae has been reported earlier [23]. The optimal dose of CTAB required to eradicate the grazers without affecting the microalgal growth was determined as 3 µM for *Brachionus*, 2 – 4 µM for *Sterkiella* and 3 µM for *Paraphysomonas*. These concentrations resulted in complete eradication of grazers within 24 – 48 h. At the same time, the optimal dose of CTAB resulted in a decrease in daily mean biomass losses to 8.6% for *Chlorella* contaminated with *Brachionus*, 6.1% for

Chlamydomonas contaminated with *Sterkiella* and 1.5% for *Chlamydomonas* contaminated with *Paraphysomonas* **(Fig. 1).**

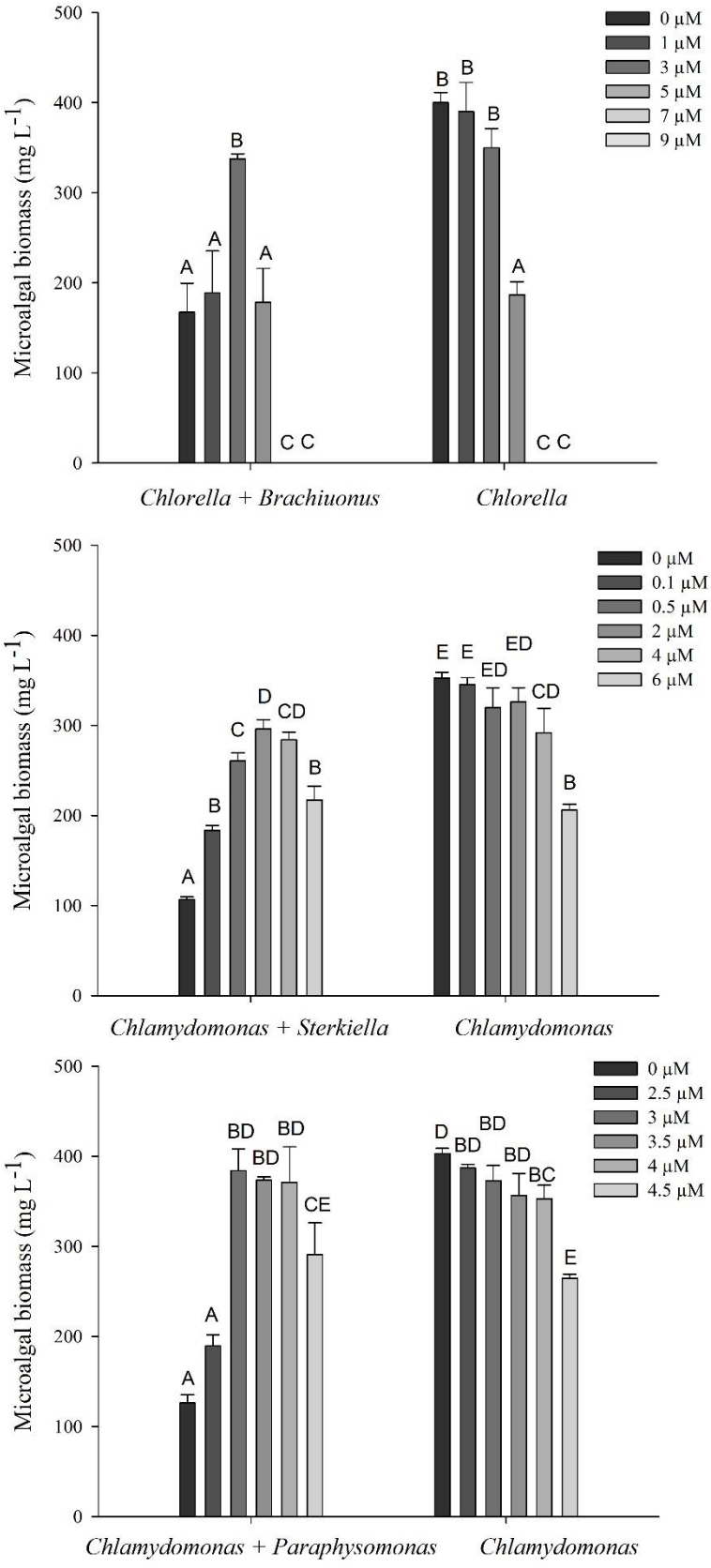


Figure 1: Microalgal biomass (dry weight, mg L^{-1}) in grazer contaminated (left) and uncontaminated microalgal cultures (right) treated with different doses of CTAB at the end of each growth experiment. For each microalgae-grazer combination five different CTAB concentrations were applied. Two-way ANOVA

was used to test for the effects of CTAB concentration, grazer presence and their interaction on microalgal dry weight biomass (mg L^{-1}) at the end of each experiment. Statistically different microalgal biomass concentrations for each tested grazer ($p<0.05$, n =12, two-way ANOVA with Tukey HSD posthoc test) are indicated by a different letter. Each data point is the mean of three replicates. Error bars denote one standard deviation. If no bar is shown, microalgal biomass concentration equals zero.

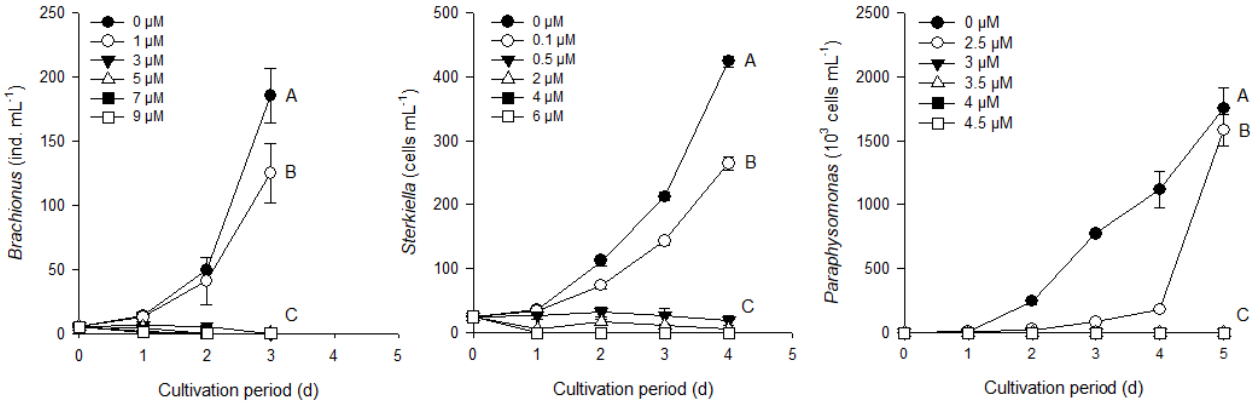


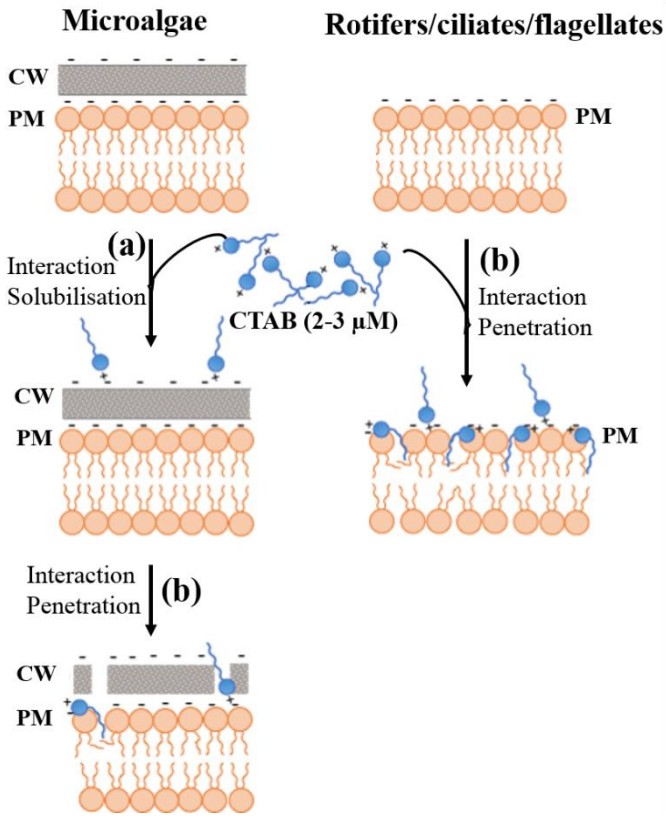
Figure 2: Influence of CTAB concentration on growth of the rotifer *Brachionus*, the ciliate *Sterkiella* and the flagellate *Paraphysomonas*. Each grazer species was exposed to five different CTAB concentrations and compared with a control culture (= 0 µM). One-way ANOVA was used to test for the effect of CTAB concentration on grazer abundance at the end of each experiment. Statistically different values ($p<0.05$, n= 6, One-way ANOVA with Tukey HSD post hoc test) are indicated by a different letter. Each data point is the mean of three replicates. Error bars denote one standard deviation.

Application of CTAB at the optimal dose to cultures without grazers caused similar daily mean biomass losses: 6.9% for *Chlorella* cultures at 3 µM and 2.1 – 2.8% for *Chlamydomonas* cultures at 2 - 3 µM. However, these losses were much lower and trivial when compared to losses caused by grazers in the contaminated cultures without CTAB. The microalgal biomass productivity at these optimal doses was significantly higher ($p<0.05$) compared to contaminated cultures treated with lower and higher doses of CTAB (**Fig. 1**). These findings indicate that if CTAB is applied with the optimal dose it can protect microalgae biomass from devastating grazers and prevent a culture crash, with only a limited reduction in biomass productivity.

The CTAB dose needed to kill 50% of the grazers in 24 h (24-h LD_{50}) was estimated as, 3.7 ± 0.55 µM (1.31 ± 0.2 mg L^{-1}) for *Brachionus*, 1.98 ± 0.06 µM (0.69 ± 0.02 mg L^{-1}) for *Sterkiella* and 2.7 ± 0.04 µM (0.98 ± 0.01 mg L^{-1}) for *Paraphysomonas*. The 24-h LD_{50} for the two microalgae species was

12.14 ± 0.24 µM (4.42 ± 0.09 mg L^{-1}) for *Chlorella* and 5.41 ± 0.09 µM (1.96 ± 0.03 mg L^{-1}) for *Chlamydomonas*.

These results indicate that CTAB negatively affects both microalgal grazers and microalgae. Yet, microalgal grazers are more sensitive to CTAB than microalgae, and there is also a difference in sensitivity to CTAB between microalgal species. This difference may be caused by an interaction between the action mechanism of CTAB and the differences in the cell structure of the microalgae and their grazers **(Scheme 1)**. The likely mechanism by which CTAB interacts with living cells is by (a) solubilizing cell wall polysaccharides and/or (b) destabilizing the lipid bilayer of the plasma membrane [24,25]. Thus, CTAB is capable of affecting the organisms in spite of having a protective cell wall. However, organisms with a cell wall can be expected to be less impacted by quaternary ammonium surfactants such as CTAB, when compared to organisms lacking a cell wall [15]. Most microalgal grazers either lack a cell wall (rotifers, ciliates) or have a cell that is covered by loose silicaceous scales (the flagellate *Paraphysomonas*). This allows CTAB to directly interact with their cytoplasm membrane, which should rapidly result in loss of cellular integrity. On the contrary, the cell wall of most microalgae makes them less prone to the destructive action of CTAB, at least at concentrations (< 4 µM) at which the grazers lacking a cell wall are susceptible. The difference in susceptibility between the microalgae is likely in part due to differences in algal physiology and differences in cell wall composition and structure [15,26]. This difference in CTAB vulnerability between grazers and microalgae is a necessary prerequisite for successful application of CTAB for grazer control in microalgal cultures.



Scheme 1: Illustration of the possible action mechanism of CTAB on microalgae and grazers, owing to their different cell coverings, which have been exploited in the current study to selectively eradicate grazers from the microalgal culture (derived from [24,25]). In case of microalgae, the CTAB acts on the cells via a two-step process (a) interaction of positively-charged head group with negatively-charged cell wall surfaces and solubilisation of cell wall polysaccharides to create localized pores; (b) interaction of the positively-charged head group with negatively-charged phosphate moiety of phospholipid bilayer and penetration of the tail group of CTAB inside the bilayer to interact with the lipidic tails. This alters the phospholipid assembly, thereby causing membrane disruption and results in leakage of intracellular components and cell death. Since the grazers lack the cell wall, CTAB directly acts on the plasma membrane following the step (b) and affects them quicker than the microalgae. CW, cell wall; PM, plasma membrane.

3.2. Practical implications of using CTAB as pesticide in microalgal cultivation

Quaternary ammonium salts like CTAB are widely used as disinfectants against bacteria and fungi. The results of this study show that CTAB is also effective against a range of grazers that contaminate microalgal cultures. The dose at which CTAB kills microalgal grazers (2 - 3 µM) is an order of magnitude lower than the doses used to kill bacteria or fungi (44 µM to mM range; [16]). It would be interesting to test whether CTAB is also effective against other types of contaminants in microalgal cultures, such as microscopic fungi (e.g. chytrids) or parasitic amoebae which are also a widespread

problem in microalgae production [2,3]. In this study we have only tested the effect of CTAB in fresh water microalgal cultures. However, it would be compelling to test if CTAB is also effective against grazers in industrially relevant marine microalgal cultures such as *Dunaliella* or *Nannochloropsis* spp.

CTAB has already been widely applied in microalgal processes, either as a flocculant or as an agent for cell disruption [27]. However, concentrations used for these applications are three orders of magnitude higher (0.5 – 5 mM) than the concentrations used in this study (2 – 3 µM). Hence, at the concentration range used to eradicate the grazers, no flocculation could be observed at all.

With an effective dose of 3 µM, about 110 g of CTAB would be required to treat a 100 m^3 grazer contaminated microalgal raceway pond. With an estimated cost of 10 \$ kg^{-1} CTAB, the cost for eradication of the tested grazers in 100 m^3 ponds varies from 0.7 to 1.3 \$ (Table 1). In comparison with previously reported chemical control agents such as rotenone and toosendanin, the effective CTAB dose is ~ 10 to 500-fold higher [11,28,29]. Although, rotenone and toosendanin are effective at very low concentrations, their cost kg^{-1} is ~ 7.5 to 250-fold higher compared to CTAB, which makes the application cost to a 100 m^3 algal pond similar or higher compared to CTAB (**Table 1**). In comparison with quinine sulfate and ammonium bicarbonate, the effective CTAB dose is ~ 14 to 1000-fold lower [29,30]. The higher dose needed in combination with the higher cost of ~ 20 \$ kg^{-1} for quinine sulfate makes this chemical ~ 25 times more costly to apply to a 100 m^3 culture suspension. Ammonium bicarbonate is the cheapest chemical ~ 0.11 \$ kg^{-1}, but the higher doses needed result to a similar or higher cost compared to CTAB when applied to a 100 m^3 pond (**Table 1**). This implies that the use of CTAB might be a cost competitive option compared to previously reported chemical control agents if a single application is sufficient to control grazers. The actual chemical cost during operation may deviate due to differences in persistence of the chemical in the culture medium. Environmental factors including pH, temperature and light may affect chemical degradation rates and force multiple applications of the chemical to control the grazers [30,31].

Table 1: Comparison of selected chemical control agents to CTAB used to eradicate grazers from microalgal cultures. Effectiveness of these chemicals to various grazers is reported from available literature. Chemical cost ($ kg^{-1}) and cost for application to a 100 m^3 pond for each chemical are reported. The cost of CTAB, rotenone and quinine sulfate with a minimum purity level of 98 % were assumed based on Alibaba.com price rates (march 2019). Cost of toosendanin and ammonium bicarbonate were calculated from [29] whereby the purity of ammonium bicarbonate was 99.9 % and a minimum purity of 98 % for toosendanin was assumed.

Chemical control agent	**Grazer**	**Microalgae**	**Effectiveness (24-h LD_{50}: mg L^{-1})**	**Chemical cost (US dollar kg^{-1})**	**Cost applied to a 100 m^3 pond (US dollar)**	**Ref.**
Cetyltrimethyl ammonium bromide (CTAB)	Rotifer *Brachionus calyciflorus*	*Chlorella vulgaris*	1.31	10	1.3	This study
	Ciliate *Sterkiella*	*Chlamydomonas reinhardtii*	0.69		0.7	
	Flagellate *Paraphysomonas*	*Chlamydomonas reinhardtii*	0.98		1	
Rotenone	Rotifer *Brachionus calyciflorus*	*Chlorella kessleri*	29.2 x 10^{-3}	75	2.2	[12]
	Rotifer *Brachionus manjavacas* and *Brachionus rotundiformis*	*Nannochloropsis oculata* and *Tetraselmis suecica*	71-138 x 10^{-3}		5.3-10.4	[28]
Toosendanin	Ciliate *Stylonichia mytilus*	*Chlorella pyrenoidosa*	8 x 10^{-3}	2500	2	[29]
	Rotifer *Brachionus plicatilis*	*Chlorella* and *Nannochloropsis* sp.	2.1 x 10^{-3}		0.53	[11]
Ammonium bicarbonate	Ciliate *Stylonichia mytilus*	*Chlorella pyrenoidosa*	1 x 10^3	0.11	11	[29]
Quinine sulfate	Rotifer *Brachionus calyciflorus*	*Chlorella kessleri*	14	20	28	[30]

In terms of safety, CTAB is listed in the Hazardous Materials Identification System as a level 2 substance, implying that it might cause temporary or minor injury [32]. Hence, handling of CTAB requires precautions to avoid contact with skin and eyes, and to avoid inhalation of vapour or dust. CTAB along with other QACs is widely used in domestic and industrial products and can be present in high concentration in cosmetic products such as shampoo. However, their release into the environment poses a significant risk, especially in the context of emergence of microbial resistance to other antimicrobial agents [33]. Hence, the possible environmental impact of using CTAB or other QACs in microalgal systems has to be carefully assessed. This report is aimed to demonstrate the technical feasibility of using CTAB as a control agent against three types of grazers readily contaminating microalgal cultures. However, before effective application of CTAB, it is essential to understand how the culture conditions affect the persistence and degradation, if any, of CTAB during the microalgal cultivation process.

4. Conclusions

Low concentrations of CTAB ($\leq$ 3 µM) can be used to effectively eradicate microscopic grazers from microalgal cultures, including rotifers, ciliates and flagellates. Although CTAB has negative effects on microalgae at higher doses, application of CTAB at an optimal dose to a contaminated microalgal culture can avoid large losses in biomass productivity. Thus, CTAB has the potential to serve as a low-cost, fast-acting control agent for the extermination of various types of grazers. However, additional environmental testing and biodegradability of CTAB needs to be assessed before practical application in large-scale cultivation systems.

Supplementary Information of this work can be found in online version of the paper.

Acknowledgements

This research was financially supported by the Research Foundation Flanders (FWO Ph.D. Fellowship B. Deruyck). Nguyen Thi Kim Hue was supported by a PhD scholarship provided by the Ministry of Education and Training of Vietnam. Ramasamy Praveenkumar received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement no 751637.

All the authors declare that there are no conflicts of interest. No informed consent or human or animal rights are applicable.

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