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Prevention of algaculture contamination using pesticides for biofuel production

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ABSTRACT

Compared to terrestrial biofuels, algal biofuels have the advantages of high areal productivity, high oil content and the ability to be cultivated using non-potable water on non-arable land. However, just like terrestrial agricultural crops, algae can be grazed by a wide variety of organisms, particularly in open pond systems. Algal grazers can be controlled using pesticides to make a more sustainable source of biofuels. In this study, we choose *Chlorella kessleri* as the model freshwater algal producer and select the freshwater rotifer, *Brachionus calyciflorus* and the freshwater ciliate, *Colpoda* sp. as the model algal grazers. The pesticides - quinine (QN), niclosamide (NC), 3-trifluoromethyl-4-nitrophenol (TFM), rotenone (ROT) and protons (pH) are used to inhibit algal grazers. *C. kessleri* is not affected by TFM and NC at concentrations up to 39 and 31 μM respectively, while inhibition of *B. calyciflorus* is achieved at concentrations of 18 and 14 μM respectively. *Colpoda* sp. is inhibited by NC at 8 μM, while, QN, TFM and ROT inhibit *Colpoda* sp. at 30 μM. A pH range between 8.4 and 10.3 inhibits *B. calyciflorus* and allows *C. kessleri* to grow well. Compared to previous results using ROT, the findings presented in this study demonstrate that algal grazers may be effectively controlled by using QN, NC and TFM yet may require higher doses than ROT.

1. Introduction

Algae are a renewable source of biodiesel due to their high growth rates, high oil content and the ability to be grown using brackish and non-potable water on non-arable land. However, algae are primary producers and are preyed upon by a variety of grazers resulting in frequent algal pond 'crashes'. Predation by algal grazers has not been well quantified, but it is estimated to be 10–30% for open ponds [1]. Rotifers, amoeba, fungi, ciliates and other organisms were observed to contaminate ponds in the Algae Testbed Public, Private, Partnership (ATP^3) project [2]. Depending on temperature, freshwater algae cultures could not be sustained for > 2–3 weeks at all testbeds before significant contamination occurred whereby ponds had to be cleaned and re-inoculated.

Common grazers are ciliates and the zooplankton groups, Rotifera (rotifers), and a subclass of Crustacea, Copepoda (copepods) as well as the cladocerans (water fleas). All these taxa have fast growth rates and are capable of decimating algae mass cultures in a few days [3,4]. Compared to mechanical methods, chemical treatment may be a better method to remove rotifers [5]. Rotifers and ciliates are well-known algae grazers and are good model organisms for this study due to their microscopic size, genetic uniformity, and high sensitivity to toxic chemicals [6,7]. Rotifers have more offspring than ciliates and grow much faster, reaching densities of 500,000 rotifers per liter in nutrient dense environments [5].

Ciliate contamination has been recorded in outdoor algal cultures of *Dunaliella salina* within 48–72 h [4]. Rotifers and ciliates are animals, live in both fresh and salt water, and are model algal grazers because of their ubiquity, small size, population uniformity, ease of cultivation, sensitivity to pesticides, and high reproductive rates [4,5,8–10]. Thus, in this research, *Brachionus calyciflorus*, a freshwater rotifer, and *Colpoda* sp. were used as a model's algal grazer to investigate an efficient means to prevent algal pond crashes.

A key to algal crop protection is treating algae cultures with

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chemicals that are differentially toxic to the grazers, yet harmless to the algae at the treatment concentrations. The problem facing algae culturists is to match the right control agent to the algae species being mass cultured since there are dozens of algae species and hundreds of grazing species. Because cladocerans and copepods are arthropods, they are susceptible to a variety of insecticides developed to protect terrestrial agricultural crops with selective toxicity [11]. Rotifers are quite different phylogenetically, so their toxicity profiles require quite a different suite of compounds to control their populations. Another aspect of finding effective control agents for algal crops is that some traditional compounds like copper [12] and hypochlorite [13] are difficult to dose properly to achieve selective grazer toxicity because they are rapidly degraded or adsorbed by the algae, reducing their bioavailability to grazers. The challenge is to find compounds that are selectively toxic to grazers, easy to apply, not detoxified by the algae, not environmentally persistent upon discharge, and cost-effective at the treatment doses required.

Several chemicals are known to reduce rotifer populations including mercury, hypochlorite, zinc, cadmium, copper and aluminum [14]. Within a specific time period, the LC_{50} is known as the chemical concentration lethal to 50% of a test population. In previous work, the copper LC_{50} concentration to the rotifers *B. rubens*, *B. calyciflorus* and *B. plicatilis* were 26, 19, and 120 ppb, respectively [6,12–15]. The hypochlorite LC_{50} concentration in the three rotifers *B. calyciflorus*, *B. rubens*, and *B. plicatilis* was 26, 19, and 120 ppb, respectively. Both copper and hypochlorite were deemed relatively ineffective due to the adsorption by algae, which decreased the bioavailability of copper and attenuation by the nonselective reduction of hypochlorite [12,13].

Quinine (QN) was first used to prevent malaria by inhibiting glycolysis - the synthesis of protein and nucleic acids in *Plasmodium sp.*, a protozoan parasite. QN is a schizonticidal agent because it targets rotifers which reproduce schizogony or asexually [16,17]. In ciliates, QN blocks potassium channels which disrupts osmotic regulation [4]. The QN LC_{50} for *B. calyciflorus* was found to be 17 μM while *C. kessleri* was uninhibited at QN concentrations < 25 μM [18]. The 24-h QN LC_{100} for *Colpoda* sp. was found to be 37–43 μM, whereas the 72-h QN EC_{50} for *Dunaliella salina* was 45 μM [4].

The most effective pesticides against algal grazers may be those that disrupt respiration – i.e. the electron transport chain (ETC). Algae are insensitive to ETC disruptors because they have alternative electron transport pathways [19]. Rotenone (ROT) is a structural homologue to ubiquinone in Complex I of the ETC and was shown to effectively inhibit rotifers in co-culture with algae [20]. Temperature, light, pH and cultivation media have been shown to affect rotenone persistence [21]. It can persist as long two weeks or more, which is a sufficient time to control grazers in algae ponds [9,21,22].

Niclosamide (NC) acts by uncoupling oxidative phosphorylation and, thus, may inhibit algal grazers [23]. NC inhibits cestodes, flatworms and snails which are intermediate hosts for *Schistosoma sp.* [24]. NC is highly toxic to fish and is applied to manage fish ponds [25]. It is attenuated by UV light and has a short half-life [26].

Although the piscicide, 3-trifluoromethyl-4-nitrophenol (TFM), has been used to reduce sea lampreys invading the Great Lakes, its toxicity against algal grazers is less known [27,28]. TFM is similar to phenolic compounds and impairs mitochondrial ATP production and will target all organisms that respire. Earlier researchers have studied TFM toxicity, in both target and non-target organisms, especially fish larvae [27,29–31]. Previous studies have described ponds containing algae as treated with TFM at concentrations typically used to control lampreys. These TFM-treated ponds demonstrated small decreases in dissolved oxygen (DO), which indicates TFM had an insignificant effect on algae. TFM is very soluble in water with a solubility limit of 5 g/L. TFM is ionized at the pH of most natural waters, does not partition to the sediment and is resistant to biodegradation under aerobic conditions [32]. TFM persistence is well known and is a function of biochemical oxygen demand and DO [33].

This paper presents the latest experimental results using QN and the ETC-disruptors – NC, TFM and ROT to protect algacultures against rotifers and ciliates and to find the ideal concentrations that will significantly eliminate rotifers without having an ill effect on algae growth. In addition, the effect of different pH levels on rotifers were tested. In brief, separate chemical toxicity tests were conducted using 1) *Brachionus calyciflorus*, 2) *C. kessleri* and 3) a co-culture of *Brachionus calyciflorus* and *C. kessleri* to see if the algae reduce the toxicity of chemicals towards the rotifer. Thus, this study will summarize the ideal concentrations of chemicals that will reduce grazers without a substantial effect on algaculture growth and provide algae farmers with a vital strategy to control open pond crashes.

2. Materials and methods

2.1. Model algae and grazer preparation

For a complete description of cultivation media, methods and collection of the model algae *C. kessleri* (UTEX #2228) and model rotifer *Brachionus calyciflorus*, please refer to [20]. *Colpoda* sp. was isolated from mass cultures of the freshwater rotifer *B. calyciflorus* at the Georgia Institute of Technology. *Colpoda* appeared spontaneously as a contaminant in these cultures from sources unknown. *Colpoda sp.* was cultured in artificial seawater (ASW) and was prepared from Instant Ocean salts (Aquarium Systems, Mentor, Ohio) and deionized water at 10 ppt. ASW was used as dilution water to prepare a medium called MYE, which consists of 50 mg dried skim milk and 100 mg dry baker's yeast in 100 mL ASW at 10 ppt. This MYE stock medium was diluted 1 to 100 with ASW before inoculating with ciliates. Ciliate cultures were maintained in an incubator at 28 °C in the dark.

2.2. 3-Trifluoromethyl-4-nitrophenol (TFM) and niclosamide (NC) toxicity tests using B. calyciflorus

Toxicity evaluation methods of rotifers by pesticides have been conducted in spring water via 24-well polystyrene plates at 25 °C as standardized by Snell et al. [34]. Briefly, chemical stock solutions (TFM and NC) were prepared using dimethyl sulfoxide considering water solubility limits of these chemicals of approximately 5 and 3 g/L. The working stocks were kept at −20 °C. Multiple experiments were conducted at 5 different concentrations of TFM (ranging from 0.2 μM to 97 μM) plus control, and NC concentrations (ranging from 0.2 μM to 57 μM) plus control, in 24-well plates at 1 mL per well and closed to reduce evaporation. Around 1 mL of rotifer culture (containing around twelve rotifer neonates) was transferred into each well and were kept at 25 °C for 24 h. In each trial, rotifers were counted in triplicate by viewing 1 mL samples using a stereomicroscope and rotifer mortality was determined after 24 h to determine LC_{50}'s. The rotifers not moving for 7 s were regarded as dead. All trials were conducted in triplicate.

2.3. 3-Trifluoromethyl-4-nitrophenol (TFM) and niclosamide (NC) toxicity tests using C. kessleri

Based on the LC_{50} results of TFM and NC that were obtained from the rotifer toxicity experiments, the algae suspensions in Bold's basal media (BBM) medium were spiked with TFM concentrations of 0, 10, 19, 29, and 39 μM and NC concentrations of 0, 2, 12, 21, and 31 μM. The experiments were conducted under illumination condition (light path of ~48 cm), at 25 °C and 150 rpm using 250 mL Erlenmeyer flasks (100 mL of algae culture) on a platform shaker (Innova 2100, New Brunswick Scientific). Optical densities (OD) were measured in triplicate over 6 to 8 days to evaluate algal growth rate. All experiments were conducted in triplicate.

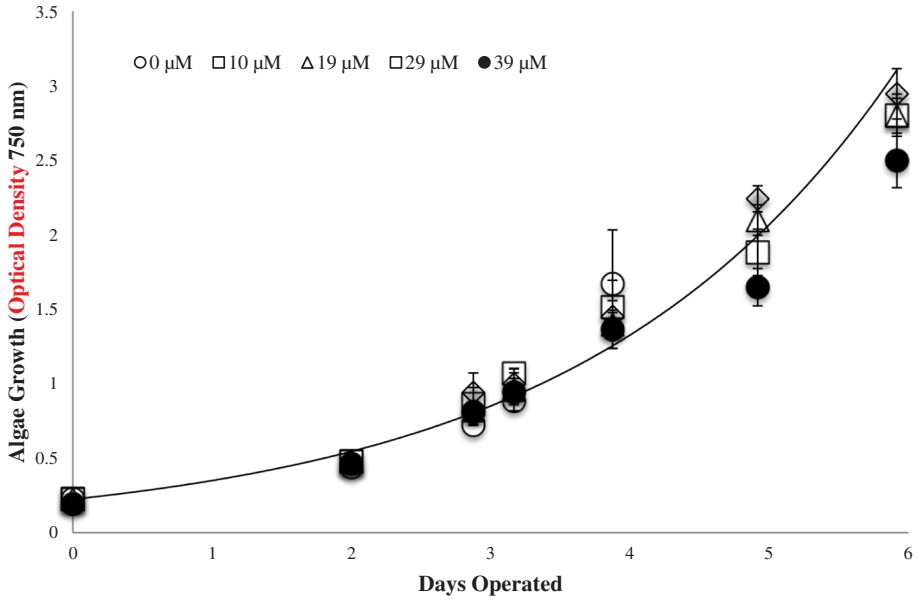

Fig. 1. Effect of various 3-trifluoromethyl-4-nitrophenol (TFM) concentrations on *C. kessleri* growth rate. Data are shown as mean ± SD, $n = 3$.

2.4. 3-Trifluoromethyl-4-nitrophenol (TFM) and niclosamide (NC) toxicity tests using co-cultures of B. calyciflorus and C. kessleri

C. kessleri and *B. calyciflorus* were co-cultured to evaluate the toxicity of TFM and NC on *B. calyciflorus* in the presence of *C. kessleri*. Suspensions of *B. calyciflorus and C. kessleri* were used to inoculate separate experiments (100 mL algal culture containing approximately 15 rotifers per 1 mL in a 250 mL Erlenmeyer flask) using different concentrations of TFM and NC; TFM concentrations ranged from 0 to 100 µM, while NC concentrations ranged from 0 to 40 µM. The experiments were conducted in triplicate exactly as described in Section 2.3 except that rotifers were added to the algae suspensions. Rotifer numbers and OD's were measured in triplicate once a day over seven days. Rotifers were sampled from the algae suspension using a 1 mL pipette tip in which the end of the pipette was cut (to make the hole slightly bigger) to make sure rotifers freely entered the tip. Suspensions were mixed by hand and 1 mL aliquots were viewed under a stereomicroscope to obtain the number of rotifers per mL.

2.5. 3-Trifluoromethyl-4-nitrophenol (TFM), niclosamide (NC), and rotenone (ROT) toxicity tests using Colpoda sp

Four experiments were conducted with three replicates to test the pesticides QN, TFM, NC and ROT on *Colpoda* sp. The LC_{50} toxicity test of different pesticides for *Colpoda* sp. were conducted in spring water using 24-well polystyrene plates at 25 °C as described by Snell et al. (1991) [34]. Different chemical agents were evaluated at concentrations (0, 2, 4, 8, 15 and 30 µM) in 24-well plates at 1 mL per well. Ten neonates of *Colpoda* sp. were transferred into each well and were incubated for 24 h. The well plates were examined under the stereomicroscope for ciliate mortality after 24 h to calculate LC_{50}'s. The numbers of live and dead *Colpoda* sp. were recorded, with *Colpoda* sp. not moving for 10 s regarded as dead.

2.6. The effect of pH on co-cultures of B. calyciflorus and C. kessleri

The effect of pH on *C. kessleri* was tested using four one-litter glass tubes (5.1 cm width and 64 cm height), and Bold's basal media (BBM) was used as the algae growth medium. The culture was given continuous illumination from eleven 40-Watt 1.2 m long cool white fluorescent light bulbs with a light path of approximately 7.6 cm, and sparged with air at ~1 L/min at room temperature (approximately 25 °C). The Cole-Parmer Model 300 pH/ORP meter (Cole-Parmer Instrument Co., Vernon Hills, IL) was used with a temperature controller, i.e., the Wahl Model C962 1/8-DIN Controller, which were connected to a solenoid; CO_2 was dosed periodically to maintain the pH at 7.2, 8.4, 9.0 or 10.3.

In separate experiments, co-cultures of *C. kessleri* and *B. calyciflorus* were conducted similarly to Section 2.4 (100 mL algal culture containing approximately 15 rotifers per 1 mL in a 250 mL Erlenmeyer flask) at the same pH range using a 1 M phosphate buffer. The pH was adjusted twice daily using 1 M HCl and KOH solutions. Rotifer counts and OD's were determined once a day over seven days as described in Section 2.4.

2.7. Data analysis

All obtained data were evaluated as means and standard deviations and then analyzed, and figures were constructed with Excel 2016, while the statistical analyses, one-way analysis of variance (ANOVA), *P*-value and IC50 were conducted using GraphPad Prism (version-8).

3. Results and discussion

3.1. Effect of 3-trifluoromethyl-4-nitrophenol (TFM) and niclosamide (NC) on B. calyciflorus and C. kessleri

Since TFM and NC are known to affect animals, and their effect on algae are relatively unknown, we tested their effect on *B. calyciflorus* and *C. kessleri*. Figs. 1 and 3 showed that *C. kessleri* was not affected by TFM and NC at concentrations up to 39 and 31 µM, respectively, and are not different than zero ($p = 0.995$ and 0.893, respectively), while the LC_{50}s for *B. calyciflorus* were achieved at concentrations of 18 and 14 µM, respectively, as shown in Figs. 2 and 4.

The TFM and NC rotifer LC_{50}s are similar to the LC_{50} of QN (17 µM) yet greater than rotenone (0.01 µM) observed in earlier work [18,20,35]. QN did not affect *C. kessleri* growth at concentrations up to 25 µM and completely inhibited *B. calyciflorus* at 7.7 µM [18].

Fig. 5 shows the effect of TFM and NC on the relative growth rate of *C. kessleri* after 144 h of exposure. TFM concentrations had a no effect on the growth rate of *C. kessleri* as the slope of the trendline was not different than zero ($p = 0.07$). Similarly, NC did not affect the growth

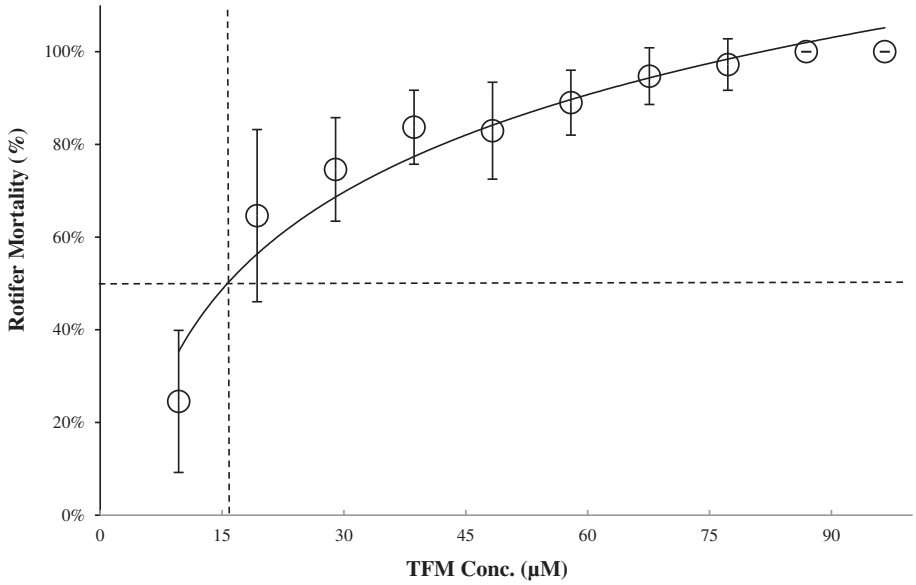

Fig. 2. Effect of 3-trifluoromethyl-4-nitrophenol (TFM) toxicity on *B. calyciflorus* mortality. The dashed lines refer to the LC50s. Data are shown as mean ± SD, n = 3.

rate of *C. kessleri* ($p = 0.24$).

The effect of TFM and NC on *B. calyciflorus* in a co-culture with *C. kessleri* were then investigated (Figs. 6 and 7). When *C. kessleri* and *B. calyciflorus* were co-cultured at TFM concentrations above ~68 μM, significant rotifer mortality was shown by 48 h. This concentration is greater than the TFM LC_{50} of 18 μM which shows that algae may have an effect on reducing TFM toxicity towards the rotifers. While the relative growth rate of *C. kessleri* was unaffected at concentrations below 68 μM, algae 'pond crashes' occurred as digested algae aggregated into clumps in the middle of the experimental flask which made obtaining accurate ODs unavailable. *B. calyciflorus* concentrations in the positive control flasks and in the experiments with TFM concentrations below 68 μM reached > 2000 rotifers per 100 mL. After Day 7, other than the aggregated algae, the supernatant was relatively clear.

When *C. kessleri* and *B. calyciflorus* were co-cultured at concentrations of NC above ~24 μM, significant *B. calyciflorus* mortality was observed by 48 h. While the relative growth rate of algae was unaffected at NC concentrations below 24 μM, algae 'pond crashes' occurred as digested algae aggregated into clumps in the middle of the experimental flask which made obtaining accurate ODs unavailable. *B. calyciflorus* concentrations in the positive controls and in the experiments with concentrations of NC below 24 μM recorded around 2000 rotifers per 100 mL. After Day 7, other than the aggregated algae, the supernatant was relatively clear.

3.2. Effect of different pesticides on Colpoda sp

Fig. 8 shows the effect of the pesticides QN, TFM, NC and ROT on *Colpoda* sp. NC completely inhibited *Colpoda* sp. at 8 μM while it took 30 μM of QN, TFM and ROT to completely inhibit *Colpoda* sp. The sharp drop in *Colpoda* sp. numbers on Day 4 is due to *Colpoda* sp. naturally encysting every four days. In a previous study [4], the effect of QN on ciliates contaminating outdoor ponds of *Dunaliella salina* were investigated and QN exhibited high inhibition of ciliates while keeping algal cells alive; 31 μM of QN prevented outdoor algal ponds contamination by ciliates.

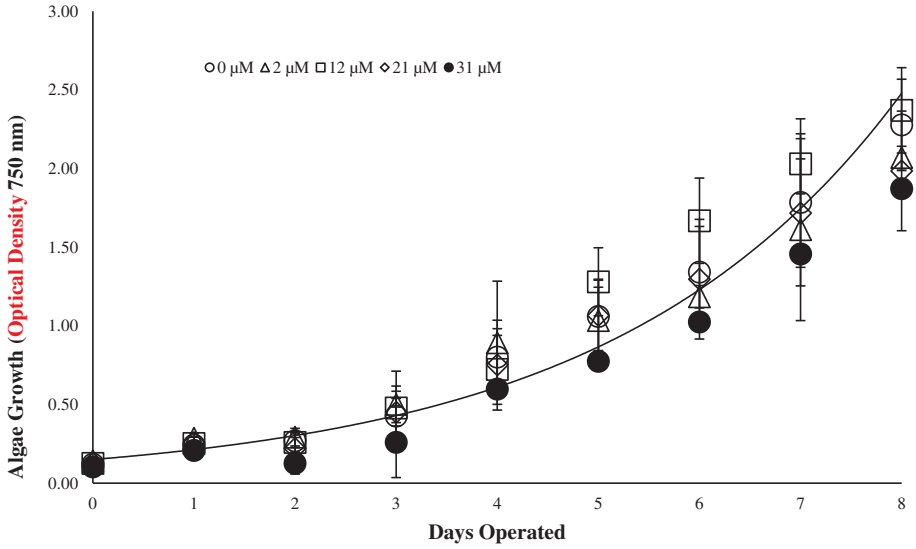

Fig. 3. Effect of various niclosamide (NC) concentrations on *C. kessleri* growth rate. Data are shown as mean ± SD, n = 3.

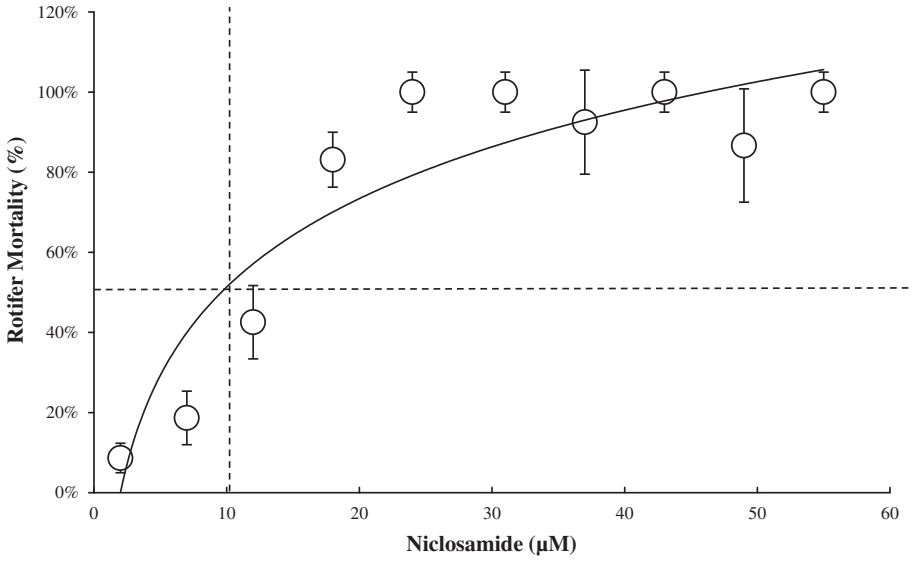

Fig. 4. Effect of niclosamide (NC) toxicity on *B. calyciflorus* mortality. The dashed lines refer to the LC50s. Data are shown as mean ± SD, n = 3.

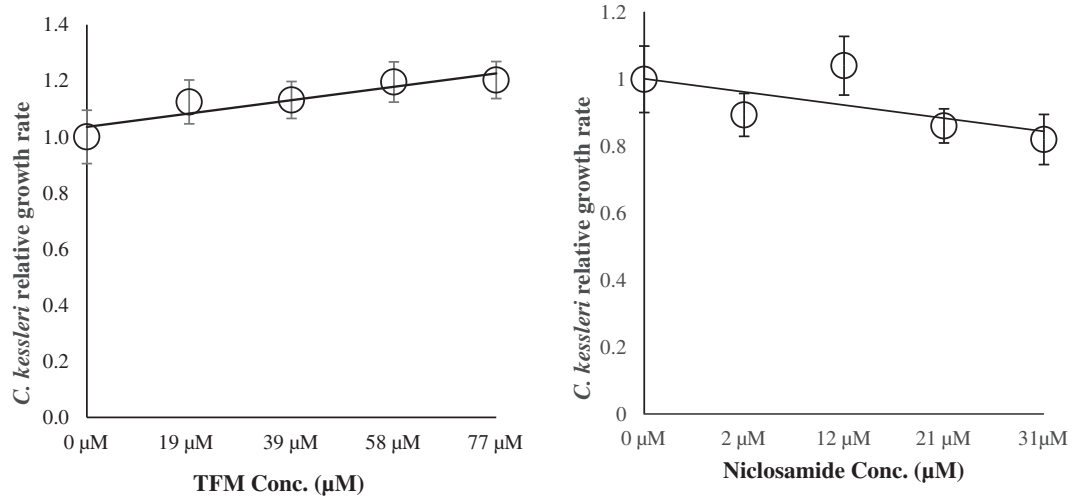

Fig. 5. Effect of 3-trifluoromethyl-4-nitrophenol (TFM) (left) and niclosamide (NC) (right) on *C. kessleri* relative growth rates. Relative growth rate (r) is calculated as $N_t = N_0 e^{rt}$, where N_t is cell density at time t, N_0 is initial cell density, and t is 6 days. Data are shown as mean ± SD, $n = 3$.

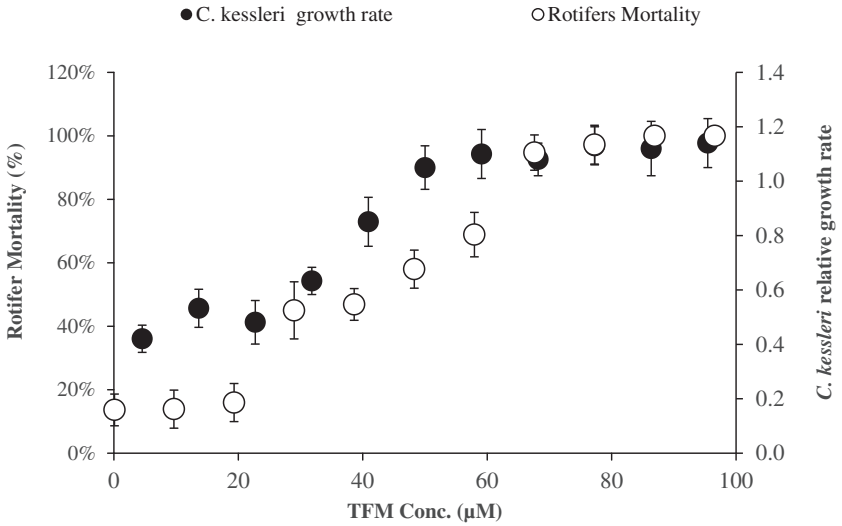

Fig. 6. Effect of 3-trifluoromethyl-4-nitrophenol (TFM) on a co-culture of *C. kessleri* and *B. calyciflorus*. Data are shown as mean ± SD, $n = 3$.

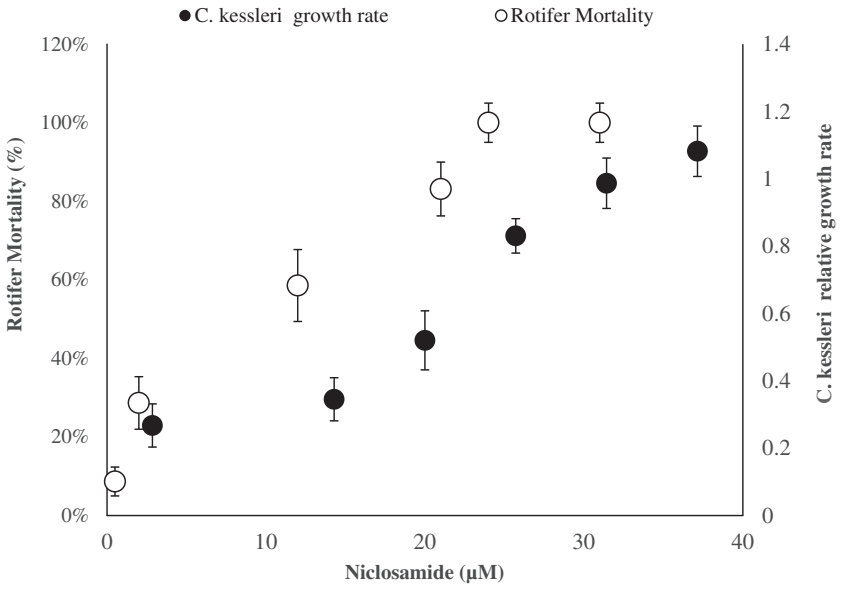

Fig. 7. Effect of niclosamide (NC) on a co-culture of *C. kessleri* and *B. calyciflorus*. Data are shown as mean ± SD, $n = 3$.

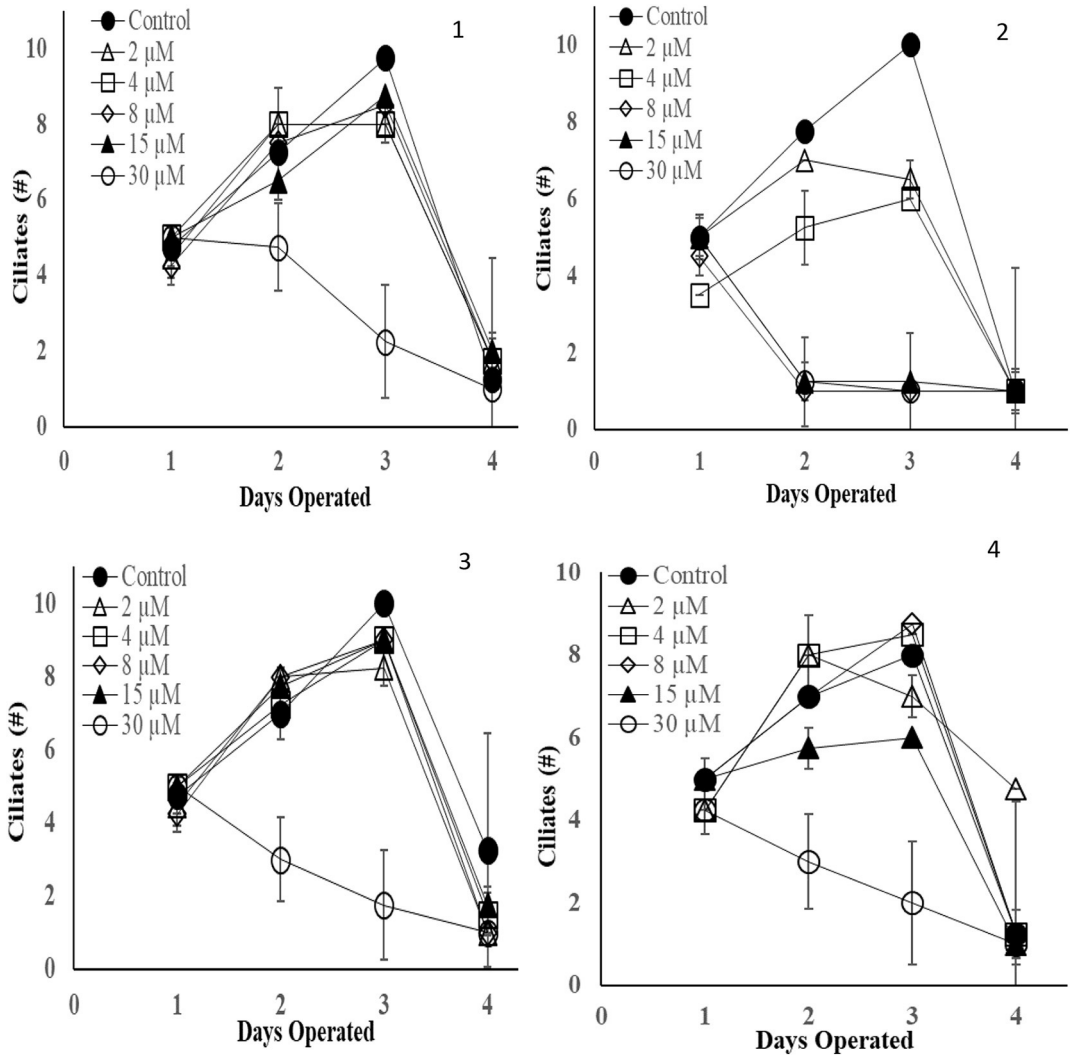

Fig. 8. Effect of different pesticides (1. quinine (QN), 2. niclosamide (NC), 3. 3-trifluoromethyl-4-nitrophenol (TFM) and 4. rotenone (ROT)) on *Colpoda* sp. Data are shown as mean ± SD, $n = 3$.

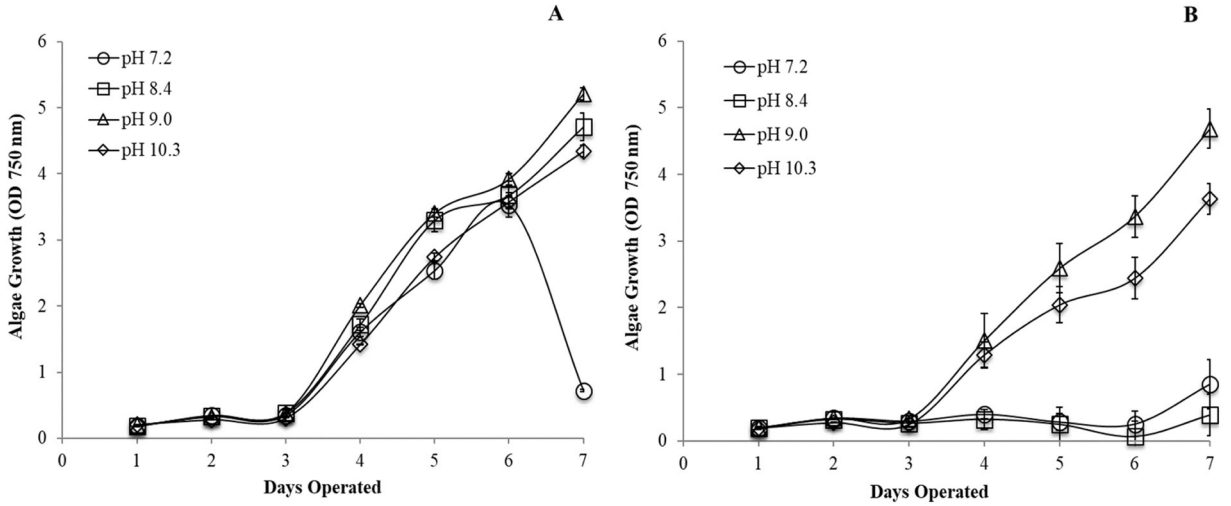

Fig. 9. Effect of pH on *C. kessleri* as a pure culture (A) and effect of pH on *C. kessleri* spiked with *B. calyciflorus* as co-culture (B). Data are presented as mean ± SD, $n = 3$.

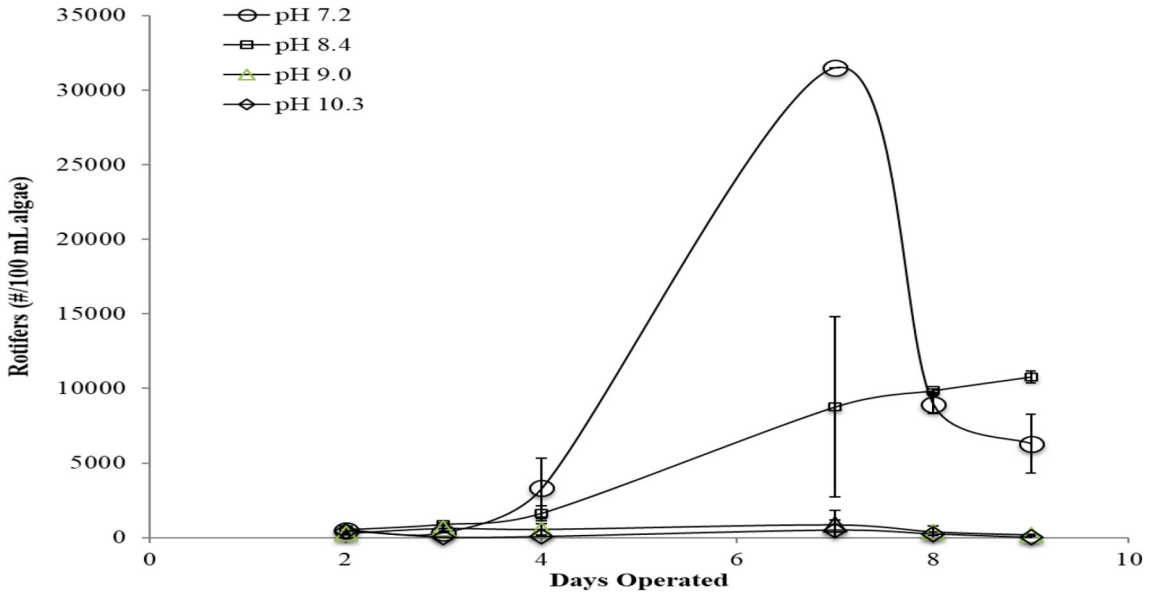

Fig. 10. Effect of pH on growth of *B. calyciflorus* cultivated on *C. kessleri*. Data are shown as mean ± SD, n = 3.

3.3. Effect of pH on B. calyciflorus and C. kessleri

Fig. 9 shows the effect of pH on *C. kessleri*-only and *C. kessleri* and *B. calyciflorus* in co-culture. Four different pH levels were tested – 7.2, 8.4, 9.0 and 10.3. In *C. kessleri*-only cultures, pH levels of 8.4 to 10.3 allowed *C. kessleri* to grow well while a pH of 7.2 inhibited growth (Fig. 9A). In co-cultures of *C. kessleri* and *B. calyciflorus*, pH levels of 7.2 and 8.4 allowed *B. calyciflorus* to grow well and 'crashed' the *C. kessleri* culture (Figs. 9B and 10). pH levels of 9.0 and 10.3 nearly completely inhibited rotifer growth. At the pH ranges tested, pH 7.2 was optimum for *B. calyciflorus* growth as reported in Fig. 10. These results show that *C. kessleri* cultures should be initiated at a pH of at least 8.4 and the pH should be allowed increase to pH 9.0 to protect *C. kessleri* cultures from *B. calyciflorus* predation. These results were confirmed in the ATP[3] freshwater ponds which were operated at pH levels of 7.8 to 8.2 and suffered significant predation [2].

4. Conclusion

Pesticides give algae farmers an inexpensive tool to control pond crashes. To keep algal biodiesel production as a sustainable source of biofuels, organisms which contaminate algal ponds need to be controlled. The pesticides QN, NC, TFM, ROT and pH were used to eliminate contaminating organisms. *C. kessleri* was not affected by TFM and NC at concentrations up to 39 and 31 μM, while *B. calyciflorus* was completely inhibited at concentrations of 18 and 14 μM, respectively, which was higher than that observed using rotenone (0.1 μM) in our previous work [20]. Furthermore, NC inhibited *Colpoda* sp. at 8 μM and QN, TFM and ROT inhibited *Colpoda* sp. at 30 μM, which shows that *Colpoda* sp. may be more resistant to rotenone than rotifers. Rotifers are filter feeders and ingest much more water, and therefore more rotenone, than ciliates. A pH range between 8.4 and 10.3 inhibits *B. calyciflorus* and allows *C. kessleri* to grow well. In conclusion, the data obtained from these experiments and results from previous work confirms that rotenone is an appropriate pesticide to control algal predation. Perhaps the best tool to inhibit contaminating organisms is to operate algae ponds at pH levels > 8.4 while dosing low concentrations of rotenone.

Declaration of authors' contributions

Authors Van Ginkel, El-Sayed, Johnston, Narode, Lee, Bhargava, Snell and Chen were involved in experimental design, data collection and analysis. Authors Van Ginkel, and El-Sayed have equal contributions of this work. We would like to thank Van Ginkel, El-Sayed, and Chen for their efforts on manuscript preparation, writing and critical reviewing. All authors discussed the results and commented on the manuscript.

Statement of informed consent

No conflicts, informed consent, human or animal rights applicable.

Statement of agreement to authorship and submission of the manuscript for review

All authors agree to authorship and submission of manuscript for review.

Declaration of competing interest

The authors are not aware of any potential conflicts of interest at the time of manuscript submission.

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