THE SELECTIVE USE OF CHLORINE TO INHIBIT ALGAL PREDATORS AND AVOID POND CRASHES FOR THE ALGAE-BIODIESEL INDUSTRY

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SUMMARY

As algae-derived biofuel is a promising renewable energy source, it is well-established that micro-algae have the potential to make a significant contribution to transportation fuel demand. Although it has many advantages including high areal productivity, there are many negative factors. One of these factors is the predation of algae by amoebas, protozoans, ciliates and rotifers, particularly in open pond systems. For example, the rotifer *Brachionus plicatilis*, is able to eat as much as 12,000 algae cells per hour and can be responsible for an entire pond crash within days. Thus, these higher organisms need to be controlled in order to satisfy large-scale algae crop and biofuel production demand.

One method of predation control involves the introduction of a toxic chemical to an algal culture that the predator has a higher sensitivity to with respect to algae. Ideally, predation could be minimized or eliminated without a substantial effect on the algal culture growth. *Chlorella kessleri* was used as the algal culture and *Brachionus calyciflorus* as the source of predation.

Research was conducted in five stages. First, chlorine dissipation tests were carried out using spring water, distilled water, Bolds Basal Medium (BBM), and three different dry weights of algal suspension in order to analyze the dissipation rate of the residual chlorine. The results showed that chlorine in distilled water and spring water rarely dissipated while chlorine concentration in algal suspension rapidly decreased by a maximum of 90% within the second hour. Second, acute chlorine toxicity tests were conducted in order to find the 24-hr LC_{50} of *B. calyciflorus*. The 24-hr LC_{50} of the test

animal was 0.198 mg Cl/L. Third, chlorine toxicity tests were conducted in order to find the LC_{50} of *Chlorella kessleri*. The 24-hr LC_{50} of *C. kessleri* was 0.321 mg Cl/L. Based on these results, the test animal was more sensitive to chlorine than the test algae; therefore chlorine may be used to avoid algae pond crashes by *B. calyciflorus.* Fourth, *C. kessleri* and *B. calyciflorus* were combined into one test to determine how long it would take to observe an algal culture crash. The result demonstrated that the higher the population of predators in algal suspension, the faster it crashed. Finally, chlorine, *C. kessleri*, and *B. calyciflorus* were combined into one test to determine what chlorine concentration and dosing interval was needed to significantly reduce predation without significantly reducing algae growth. The results of the fifth experiment showed that the effective intermittent chlorine concentration was between 0.45 and 0.60 mg Cl/L, and a short interval of chlorine dosing was effective in inhibiting rotifers in algal suspension. Even though the rotifers in algal suspension were inhibited by 0.45 to 0.60 mg Cl/L, algae growth was greatly inhibited by chlorine. In this respect, future work is needed to reduce the effect on algae by chlorine or alternative chemicals.

1. INTRODUCTION

According to the annual world primary energy consumption, total primary energy consumption in 2011 and in 2012 was approximately 12,002 million tones and 12,477 million tones oil equivalent, respectively. Renewable energy accounted for 1.3% of the primary energy consumption in 2011 while accounted for 1.9% in 2012. Fossil fuels such as oil, coal and natural gas are still dominant, but renewable green energy is increasing its share in total energy use as long as people feel concern about the depletion of natural resources and the greenhouse gases (Midilli, Dincer et al. 2006, Dudley 2013).

Algae-derived biofuel is a promising renewable energy source, and it is well-established that micro-algae have the potential to make a significant contribution to transportation fuel demand (Sharif Hossain, Salleh et al. 2008, Brennan and Owende 2010, Davis, Aden et al. 2011). Algae-derived biofuel has a number of advantages. The areal productivity of algae-derived biodiesel is almost 10 times higher than biodiesel derived from oilseed crops such as rapeseed and canola; therefore will provide less competition with traditional agriculture for available land (Schenk, Thomas-Hall et al. 2008). Furthermore, due to its high areal productivity, the water footprint compares favorably with other biofuels. The water usage of algae biodiesel in open pond and enclosed pond was 6.25 and 0.884 $L.MJ^{-1}$, respectively, while water usage of rapeseed and soybean was 409 $L.MJ^{-1}$ and canola was 31.5 $L.MJ^{-1}$ (Harto, Meyers et al. 2010, Subhadra and Edwards 2011, Batan, Quinn et al. 2013, Guieysse, Béchet et al. 2013). Moreover, water recycling in algae cultivation can reduce this water footprint by as much as 84% (Yang, Li et al. 2011, Yang, Xu et al. 2011).

However, as algae are a food source for higher organisms such as amoebas, protozoans, ciliates and rotifers, predation must be controlled. This is particularly evident in open pond systems. One of these aforementioned higher organisms, the rotifer *Brachionus plicatilis,* is able to eat as much as 12,000 algae cells per hour and can cause an entire pond to crash within days (Lubzens 1987). Thus, higher organisms need to be controlled in order to realize the advantages of algal biofuel production (DOE 2010).

One method to control predation is to introduce a toxic chemical to an algal culture that the predator has a higher sensitivity to with respect to the algae. Ideally, predation could be minimized or eliminated without a substantial effect on the algal culture growth. Rotifers are suitable animals for toxicity tests because of their microscopic size and ease of use, genetic uniformity, and high sensitivity to toxic chemicals (Dahms, Hagiwara et al. 2011). For these reasons, the rotifer, *Brachionus calyciflorus,* was used as a model organism to understand how to efficiently control predation.

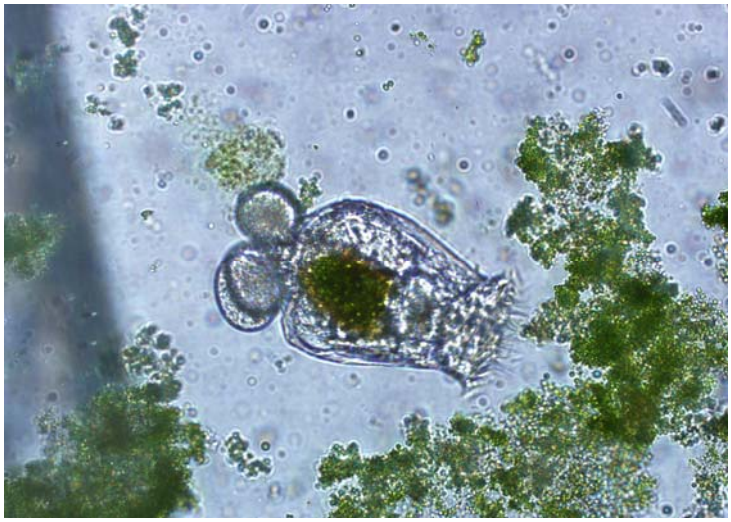


Figure 1. Rotifer, *Brachionus calyciflorus* carrying two eggs in algae culture at 20x magnification, using Olympus IX51 microscope (Olympus, Tokyo, Japan) and Olympus DP72 camera (Olympus Optical Co Ltd., Tokyo, Japan)

Hypochlorite or bleach has been widely used as a chlorine source to disinfect water. Its toxic effect on aquatic life is well documented (Zillich 1972, Roberts Jr, Diaz et al. 1975, Heath 1977, Fisher, Burton et al. 1999). However, there is little research available regarding the toxic effects of chlorine on rotifers in algal suspension. The concentration of a chemical that is lethal to 50% of a population is called the LC_{50}. In the study by Snell et al. (1991), the 1-hr LC_{50} of hypochlorite to *Brachionus calyciflorus* was 0.37 mg/L. This was substantially lower than the LC_{50}'s of Fenitrothion (6.7 mg/L) and Chlorpyrifos (12 mg/L), two commonly used insecticides (Snell, Moffat et al. 1991, Ferrando, Sancho et al. 1996). Furthermore, unlike other toxic chemicals such as heavy metals, chlorine does not leave a long lasting residue. For example, even though copper and DDT, fenitrothion and chlorpyrifos, have toxicity, they accumulate in the algae and may effect on algae growth for a long period (Lal, Lal et al. 1987, Knauer, Behra et al.

1997). These properties of chlorine, high toxic potential and natural decrement, were considered suitable for the inhibition of the rotifers in algal suspension. Chlorine can be used continuously, as needed, to keep algae predation in check. However, the rate of chlorine dissipation is unknown, especially in an algal culture where the algae itself can serve as a reductant and quickly dissipate the chlorine (Eppley, Renger et al. 1976, Sukenik, Teltch et al. 1987).

The aim of this study is to find the concentration of chlorine that will significantly inhibit rotifers without having an ill effect on algae growth. Thus, the research was conducted in five stages. First, chlorine dissipation tests were carried out using distilled water, spring water, Bolds Basal Medium and three different *C. kessleri* concentrations in order to determine the required chlorine dosing rate necessary to inhibit predation. Second, acute chlorine toxicity tests were conducted to find the 24 hr-LC_{50} of the freshwater rotifer, *B. calyciflorus*. Third, chlorine toxicity tests were conducted to find the LC_{50} of the freshwater algae *Chlorella kessleri*. Fourth, *C. kessleri* and *B. calyciflorus* were combined into one test to determine how long it would take to observe an algal culture crash. Fifth, chlorine, *C. kessleri*, and *B. calyciflorus* were combined into one test to determine what chlorine concentration and dosing interval is needed to significantly reduce predation without significantly reducing algae growth.

2. MATERIALS AND METHODS

2.1 Materials and Experimental Approach

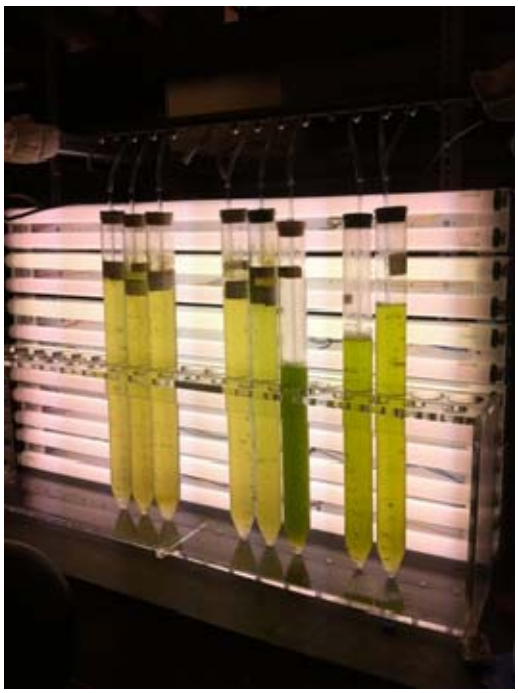
The freshwater algae *Chlorella kessleri* was obtained from the University of Texas at Austin algae collection (UTEX #2228, TX). The freshwater rotifer *Brachionus calyciflorus* was originally collected in Gainsville, Florida and it has been cultured in Dr. Snell's laboratory at Georgia Institute of Technology (Atlanta, GA). The population counts of *B. calyciflorus* were conducted using a stereomicroscope (SMZ-2T, Nikon Co., Tokyo, Japan). Bolds Basal Medium (BBM) was used as the growth media for *C. kessleri* (Kanz and Bold 1969). The composition of BBM is given in Table 1.

Table 1. BBM Chemical composition (Kanz and Bold 1969)

Chemical ingredient	mg L^{-1}
$NaNO_3$	250
$MgSO_4$ $7H_2O$	75
KH_2PO_4	175
$CaCl_2$ $2H_2O$	25
K_2HPO_4	75
NaCl	25
Vitamins*	2
Trace Metals**	2
* Vitamins Stock	mg L-1
Thiamine	200
Biotin	10
B_{12}	10
** Trace Metal Stock	mg L-1
NaFeEDTA	5,000
$ZnSO_4$ $7H_2O$	22
$MnCl_2$ $4H_2O$	180
$CoCl_2$ $6H_2O$	10
$CuSO_4$ $5H_2O$	10
$NaMoO_4$ $2H_2O$	6.4

The test chemicals, NaOCl (CAS 7681-52-9: reagent grade, available chlorine 4.00-4.99%), thiamine hydrochloride, NaFeEDTA, and $MnCl_2 \cdot 4H_2O$ were purchased from Sigma-Aldrich (St. Louis, MO). $NaNO_3$, $MgSO_4 \cdot 7H_2O$, KH_2PO_4, K_2HPO_4, NaCl, $ZnSO_4 \cdot 7H_2O$, $CoCl_2$-$6H_2O$, $CuSO_4 \cdot 5H_2O$, $NaMoO_4 \cdot 2H_2O$, and KI were obtained from Fisher Scientific (Fair Lawn, NJ). $CaCl_2 \cdot 2H_2O$ was purchased from AMRESCO (Solon, Ohio). The optical density of algal suspensions was obtained at 750 nm using a Thermo Spectronic Genesys 20 spectrophotometer (USA). Dry weight was determined using Standard Methods (Apha 1995). Reagent grade sodium hypochlorite (NaOCl) was used to make a 50 ppm stock solution for the all toxicity tests except acute toxicity test for *B. calyciflorus*.

Stock cultures of *C. kessleri* were grown in 1-L glass tubes (2" wide 2.1' tall) with a conical bottom, were given continuous illumination from the side from twelve 40-Watt 4-ft. cool white fluorescent light bulbs with a light path of approximately three inches, and sparged with air at ~1 L/min. Log-phase algal suspensions were then used to inoculate 100 mL of total batch volume, and these shake flask experiments took place in 250 mL Erlenmeyer flasks. The temperature was 25˚C and the mixing speed was 100 rpm (Platform Shaker: Innova 2100, New Brunswick Scientific). These test flasks were also continuously illuminated as stated above except the shake flasks were placed below the lights and the light path was approximately 19 inches. All glassware was soaked in 10% nitric acid, rinsed with distilled water, and autoclaved before use.



(a) *C. kessleri* Stock Culture (b) Experiments Condition

Figure 2. *Chlorella kessleri* Stock Culture and experiments environment

Rotifer eggs were hatched on the sterile petri dish containing spring water after 16 hours in the Barnstead Lab-line 305 Imperial III incubator (IL, USA), maintained at 25˚C with continuous light from 6-Watt (40W) warm white LED light bulb (2700K, 450 Lumens) with a vertical light path of approximately eight inches.

2.2 Testing Methods

2.2.1 Chlorine Dissipation Tests

Chlorine residuals were assayed by the *N*,*N*-diethyl-*p*-phenylenediamine-ferrous ammonium sulfate (DPD/FAS) titration (Eaton and Franson 2005). Spring water, distilled water, BBM, and 6.5, 72.3, and 183.6 mg/L dry weight of algal suspensions were spiked with 1 mg Cl/L at the same time and analyzed for chlorine residuals after 10,

60, 120, 180, 240 and 300 min to determine the chlorine dissipation rate. Test conditions were the same as stated above.

2.2.2 Acute Chlorine Toxicity Test of *B. calyciflorus*

Acute toxicity test methods with the freshwater rotifer, *B. calyciflorus*, have been standardized by Snell and Moffat (Snell, Moffat et al. 1991). Briefly, a 1 mg/L hypochlorite stock solution was prepared using spring water. Appropriate amounts of stock solution were immediately distributed and serial dilutions were made such that the chlorine concentrations were 0.39×10^{-4} ~ 1.280 mg Cl/L. Wells were covered to prevent chlorine evaporation. Ten to twelve neonates of rotifers were transferred into each well containing the chlorine and were incubated for 24 hours. The well plates were examined under the stereomicroscope for rotifer mortality after 24 hours to calculate LC_{50}'s. The number of live and dead rotifers was recorded by three analysts at the same time. Rotifers not moving for 10 seconds were regarded as a dead.

A total of six experiments were conducted with four replicates each at 16 different chlorine concentrations ranging from 0.39×10^{-4} to 1.280 mg Cl/L. This was followed by five experiments with four replicates each at 9 different concentrations ranging from 0.005 to 0.4 mg Cl/L.

2.2.3 Chlorine Toxicity Test of *C. kessleri*

The algae suspensions on the platform shaker were spiked with 0 to 0.45 mg Cl/L as NaOCl based on the LC_{50} obtained from the rotifer acute toxicity tests. Tests were conducted in triplicate. Optical densities were measured at 0, 24, 48, 72 and 96 hours to determine the growth rate of algae cells. The test solution pH was measured at 0 and 96 hours.

2.2.4 *C. kessleri* Pond Crash Test by *B. calyciflorus*

After incubation, rotifer neonates were transferred into test flasks containing 100 mL *C. kessleri* at a density of 43.4 mg/L dry weight with *B. calyciflorus* concentrations of 1.4, 7, and 14 neonates/mL. Tests were performed in triplicate with one algae control in triplicate. The optical densities of *C. kessleri* and the population count of *B. calyciflorus* were recorded at the same time, once a day. When counting the number of *B. calyciflorus*, 0.5 mL of well-mixed cultures were transferred onto sterile well plates and the rotifers were counted using a stereomicroscope. If the concentration of rotifers was too high to count, the suspension was diluted four times and then the number of rotifers per 0.5mL was counted.

2.2.5 The Toxicity of Chlorine on *C. kessleri* and *B. calyciflorus*

The *C. kessleri* toxicity tests above were repeated, except *B. calyciflorus* was added to determine the toxic effect of chlorine on *B. calyciflorus* in the presence of algae instead of spring water. The same shake flask procedures were used as in the pond crash tests. Three experiments with different chlorine dosing intervals and initial *C. kessleri* concentrations were conducted, in triplicate. NaOCl concentrations ranged from 0.15 to 0.75 mg Cl/L. In the first two experiments, the chlorine dosing intervals were two and six hours and in the third experiment, the dosing interval was two hours. The initial *C. kessleri* concentration was increased from 32 to 57 mg/L of dry weight in the third experiment. Using the results from the pond crash data, the initial rotifer concentration was kept constant at 7 neonates/mL. Rotifer viability was recorded once or twice per day over an interval of 96-250 hours by counting live and dead rotifers using 0.5 mL aliquot samples in triplicate.

3. RESULTS AND DISCUSSION

3.1 A Study on Correlation between Dry Weight of *C. kessleri* and Optical Density

At the beginning of the toxicity tests, a gravimetric analysis was conducted to determine the total biomass concentration at various optical densities of *C. kessleri*. Power regression for the biomass concentration in mg/L and its optical density at 750nm was fitted using SigmaPlot 11.0 (Systat Software Inc., San Jose, CA, USA) in Figure 3. The coefficient of determination, R^2, is 0.9978.

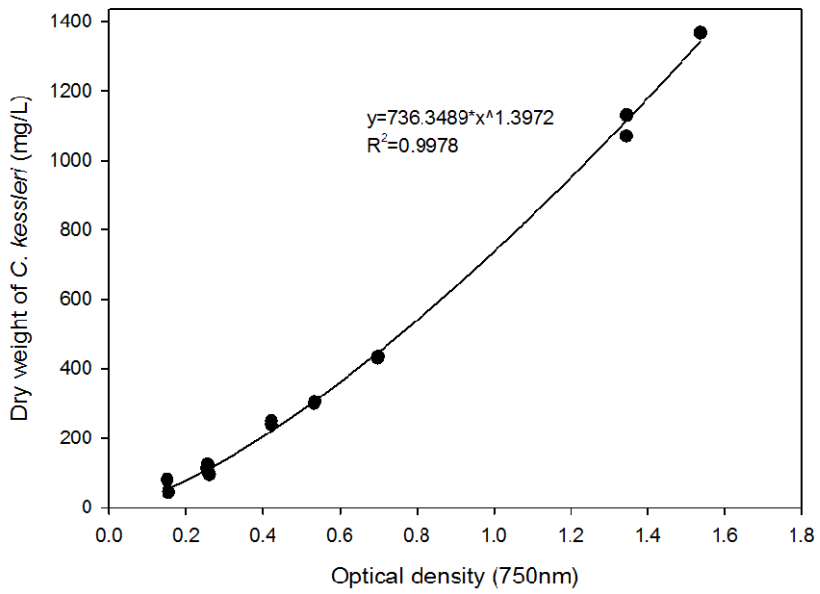


Figure 3. Dry weight of *C. kessleri* vs. Optical density at 750 nm

3.2 Results and Discussion

3.2.1 Chlorine Dissipation Tests

The chlorine dissipation rates in spring water, distilled water, BBM, and algal suspensions are presented in Figure 4. The chlorine concentration rapidly decreased in

the first hour and then gradually reduced to zero within 6 hours due to oxidation of organic compounds in the algal suspension. As the algae concentration increased from 9.1 to 183.6 mg/L, the chlorine residual decreased by 70 to 88% within the first hour and by 85 to 90% within the second hour (Figure 4). In contrast, chlorine concentrations did not rapidly decline when using distilled or spring water, and declined less when using BBM. This suggests that a reducing agent such as the organic matter in algae is needed to react with the chlorine.

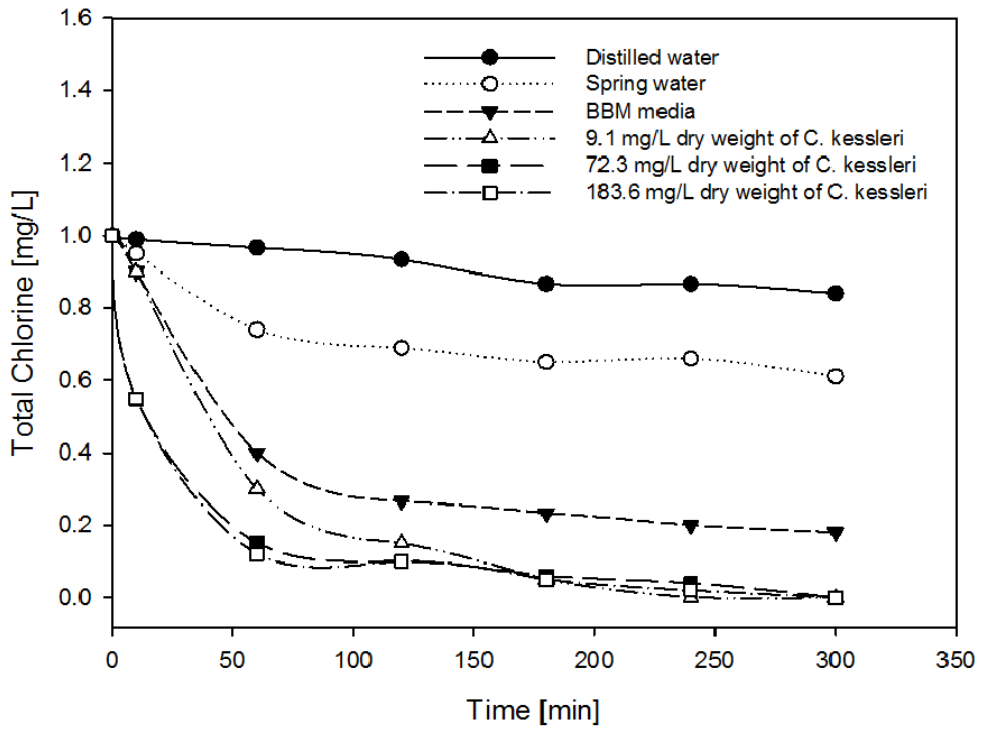


Figure 4. Chlorine dissipation rates in distilled and spring water, BBM, and three algae concentrations

3.2.2 Acute Chlorine Toxicity Test of B. calyciflorus

The 24-hrs LC_{50} for hypochlorite in spring water was 0.198 mg Cl/L (Figure 5). At 24 hours, no mortality was observed at chlorine concentrations < 0.005 mg Cl/L and

all rotifers were dead at concentrations > 0.4 mg Cl/L. The average mortality in the controls groups was 1.9% with a standard deviation of 3.9% (n = 44). Since the control mortalities were <10%, these tests can be considered consistent and the results reproducible (Snell, Moffat et al. 1991). Data were analyzed using SigmaPlot 11.0. Curve was fitted by linear regression and the equation was y = (264.734 * x) - 2.327 and R^2 = 0.951.

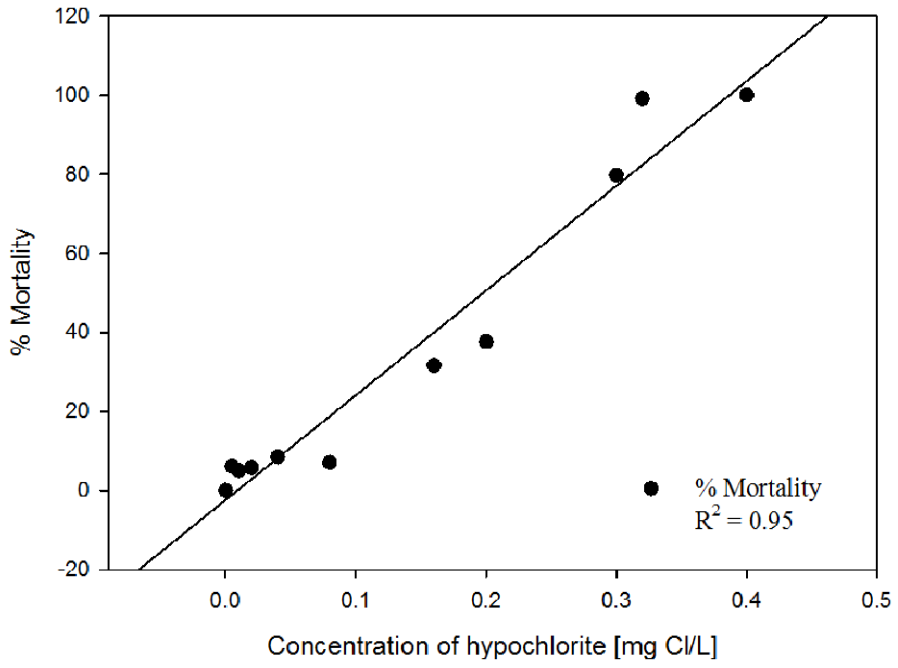


Figure 5. *Brachionus calyciflorus* 24-hrs acute toxicity tests of chlorine.

3.2.3 Chlorine Toxicity Test of *C. kessleri*

The *C. kessleri* concentrations in the controls increased from 30 to 212 mg/L dry weight within 96 hours. The twelve sets of control group growth data can be considered normal distributions. In the F-test and the Student t-test, the means and variances of twelve data sets are not statistically significant within the 95% confidence interval. The statistical analyses were performed using Matlab 7.11.0 (R2010b) (The Mathworks, Inc.).

Compared to the control, at a six-hour chlorine dosing interval, *C. kessleri* growth decreased from 9 to 49% when the chlorine concentration was increased from 0.15 to 0.45 mg Cl/L (Figure 6 and 7). The 24-h LC_{50} of *C. kessleri* was 0.321 mg Cl/L. Additionally, the LC_{50} of *C. kessleri* at 96-h was 0.477 mg Cl/L. Based on these tests, *B. calyciflorus* was more sensitive to chlorine than *Chlorella Kessleri* and chlorine may be used to avoid algae pond crashes by *B. calyciflorus.*

In these experiments, four flasks were prepared at each chlorine concentration. Three flasks were inhibited by chlorine for 96 hours while at 72 hours the fourth flask was used to measure the chlorine residual to verify the suitability of the 6 hour dosing interval. No chlorine was detected in all of these fourth flasks at 72 hours, which was 6 hours after the last chlorine dose. Thus, in later tests a 2 hour chlorine dosing interval was used.

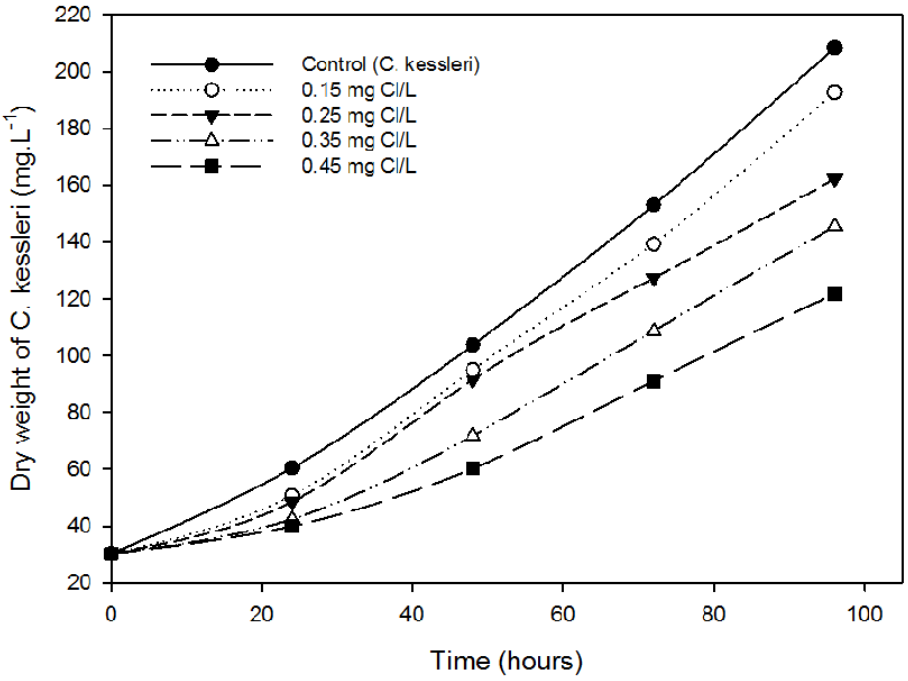


Figure 6. *C. kessleri* growth inhibition curve by 6-hrs interval chlorine dosing

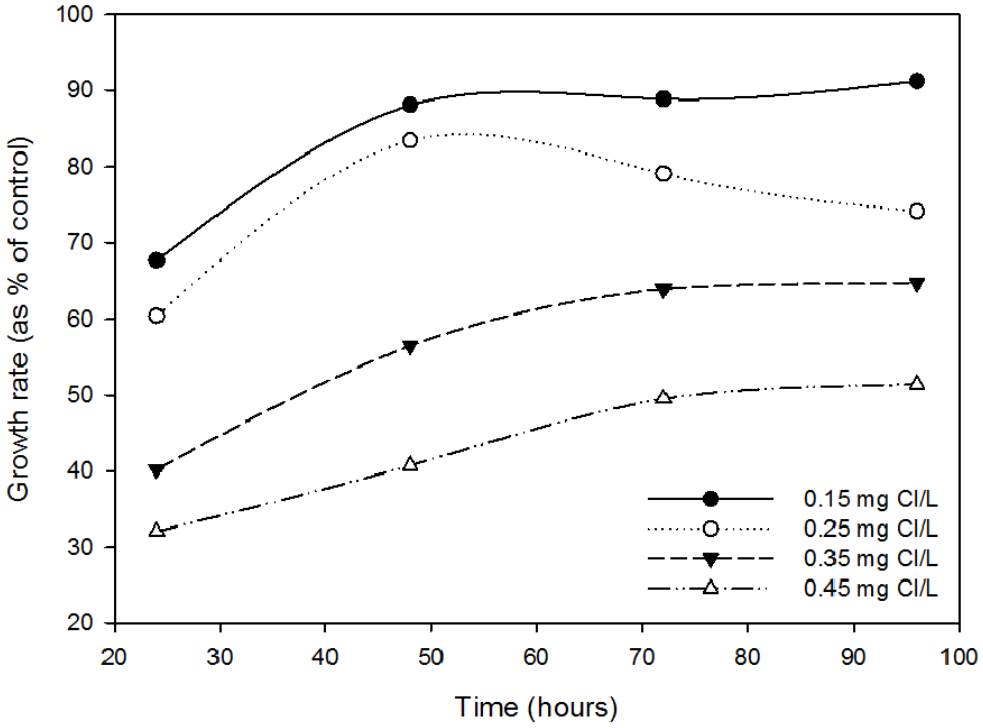


Figure 7. % of *C. kessleri* growth rates exposed to varied concentrations of chlorine

3.2.4 *Chlorella kessleri* Pond Crash Test by *B. calyciflorus*

To evaluate the *C. kessleri* pond crash rate by three different *B. calyciflorus* concentrations, 140, 700 and 1,400 neonates of *B. calyciflorus* were added into 100 mL of 43.4 mg/L dry weight *C. kessleri*. It should be noted that the number of *B. calyciflorus* added into each treatment group was an approximate value. After hatching the rotifer eggs on the petri dish, three 0.5 mL aliquot samples from the well-mixed rotifer culture were investigated by stereomicroscope to determine *B. calyciflorus* concentrations. The mean value of *B. calyciflorus* in 0.5 mL samples was 175. Consequently, 0.4 mL, 2 mL and 4 mL aliquots were added to each flask containing *C. kessleri* with exception to the control. Once the aliquoted samples of *B. calyciflorus* had been transferred to each 250-

mL Erlenmeyer test flask, the optical densities were immediately measured. Figure 8 shows the pond crash rate of *C. kessleri* by three different concentrations of *B. calyciflorus* for duration of five days.

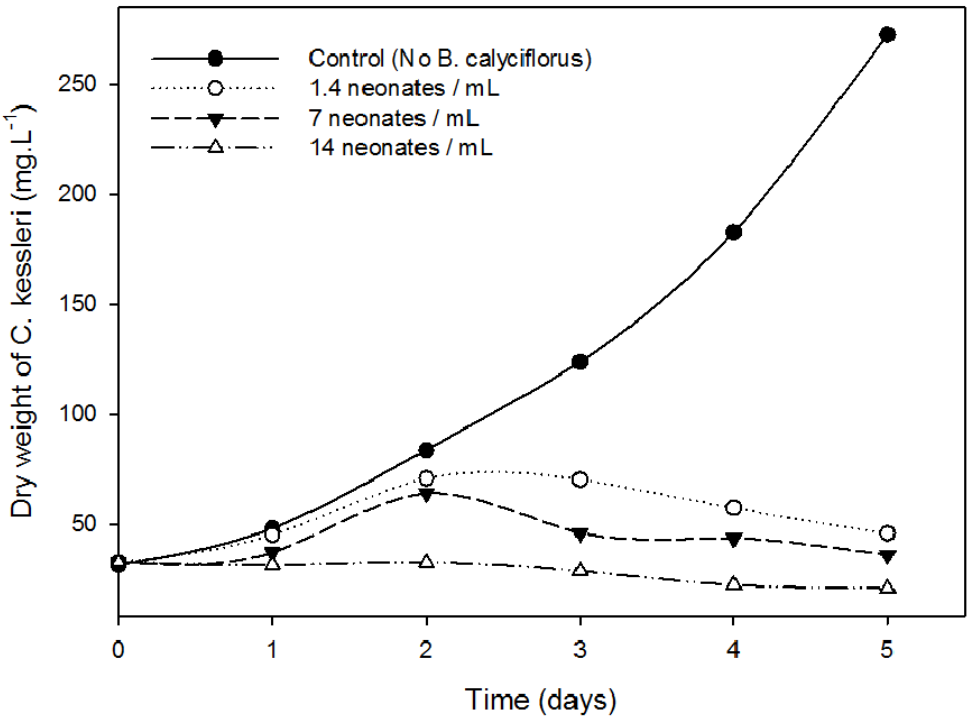


Figure 8. Pond crash curves

The dry weight of *C. kessleri* with 1.4 and 7 neonates/mL of *B. calyciflorus* increased close to the growth rate of the control until 48 hours. *B. calyciflorus* concentrations increased greatly after just one day; however, these two cultures gradually decreased until collapse. The *C. Kessleri* with 14 neonates/mL of *B. calyciflorus* did not grow due to the increased appetite demand of the high population of rotifers with respect to the amount of algal biomass present. In terms of the rate of decrease of the *B. calyciflorus* concentration from its peak, *B. calyciflorus* concentrations of 14

neonates/mL declined first, followed by those concentrations of 7 and 1.4 neonates/mL due to the limitation of prey.

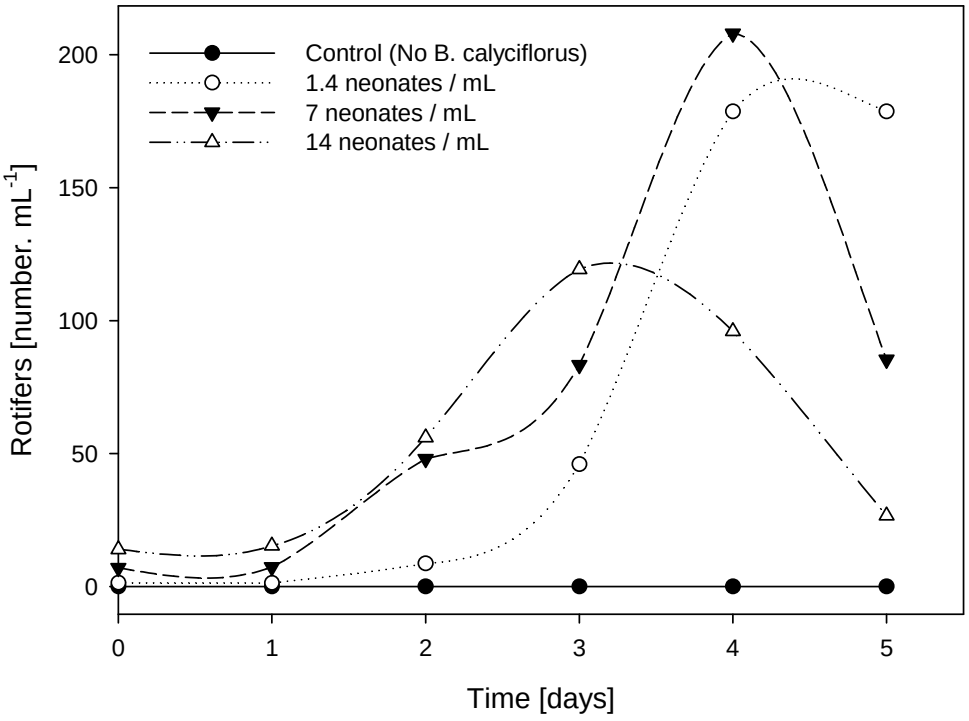


Figure 9. *B. calyciflorus* concentrations in pond crash test for 5-days

Figure 9 presents the population count of *B. calyciflorus* during the same period. Interestingly, the growth pattern of *B. calyciflorus* concentrations under these test conditions was quite consistent. *B. calyciflorus* concentrations did not increase, but maintained at a similar level for the first 24 hours. The same phenomenon was generally observed in the chlorine toxicity test on *C. Kessleri* with *B. calyciflorus*. After 24 hours, the total number of *B. calyciflorus* increased dramatically and it reached, occasionally, over 200 individuals per mL within 96 hours. Also, the algal biomass exposed to chlorine

and sufficiently high *B. calyciflorus* concentration flocculated, as presented in figure 10 (b).

(a) (b)

Figure 10. (a) *C. kessleri* normal condition, (b) C. kessleri exposed to chlorine and high concentration of *B. calyciflorus*

3.2.5 The Toxicity of Chlorine on *Chlorella kessleri* and *Brachionus calyciflorus*

According to the results from previous experiments, the toxicity of chlorine on the *C. kessleri* and *B. calyciflorus* combined environment was conducted within a range of 0 ~ 0.45 mg Cl/L to prevent a significant a drop in *C. kessleri* growth since the 24-hr LC_{50} of *B. calyciflorus* in spring water was 0.198 mg Cl/L and 24-hr LC_{50} of *C. kessleri* with 6-hours chlorine dosing interval was 0.321 mg Cl/L. The experiment ranged from 0 to 0.45 mg Cl/L, however, *B. calyciflorus* was not significantly inhibited for 96 hours by even 0.45 mg Cl/L and all test groups containing *C. kessleri* and *B. calyciflorus* eventually experienced crashing. It seems that oxidation by chorine with organic compounds in algal suspension ultimately resulted in reduced toxicity by chlorine on *B. calyciflorus*. If so, the chlorine toxicity on rotifers would not be same in spring water as

the case of rotifers existing in an algal suspension. According to Figure 4, the reaction kinetics of chlorine in algal suspension could be approximated as second order from approximately 2 hours. Therefore, chlorine-dosing interval was determined from the results of the chorine dissipation test to inhibit *Brachionus calyciflorus* in algal suspension, and chlorine concentration range was stretched to over 0.75 mg Cl/L.

Toxicity tests of chlorine on *C. kessleri* and *B. calyciflorus* consist of three experiments as presented in Table 2.

Table 2. The test conditions for toxicity of chlorine on *B. calyciflorus* and *C. kessleri*

	Dry weight of *C. kessleri* (mg/L)	*B.calyciflorus* concentration (neonates / mL)	Chlorine dosing interval
1st experiment	28	7	6 hours
2nd experiment	32	7	2 hours
3rd experiment	57	7	2 hours

In the first experiment, varied peak concentrations of chlorine were spiked into each test flask containing 28 mg/L dry weight of *C. kessleri* every 6 hours, which is the maximum duration chlorine residual can exist in the algal suspension. Then, the chlorine spiking interval was changed to 2 hours while holding constant the dry weight of *C. kessleri* in the second experiment so that a high concentration of chlorine can be maintained. In the third experiment, the dry weight of *C. kessleri* was increased from 28 to 57 mg/L while holding constant chlorine dosing interval as 2 hours to see the toxicity of chlorine on *B. calyciflorus* in sufficient algal biomass.

3.2.5.1 The First Experiment for Toxicity of Chlorine on *C. kessleri* and *B. calyciflorus*

The results of the first experiment for toxicity of chlorine on *C. kessleri* and *B. calyciflorus* are presented in Figures 11 and 12.

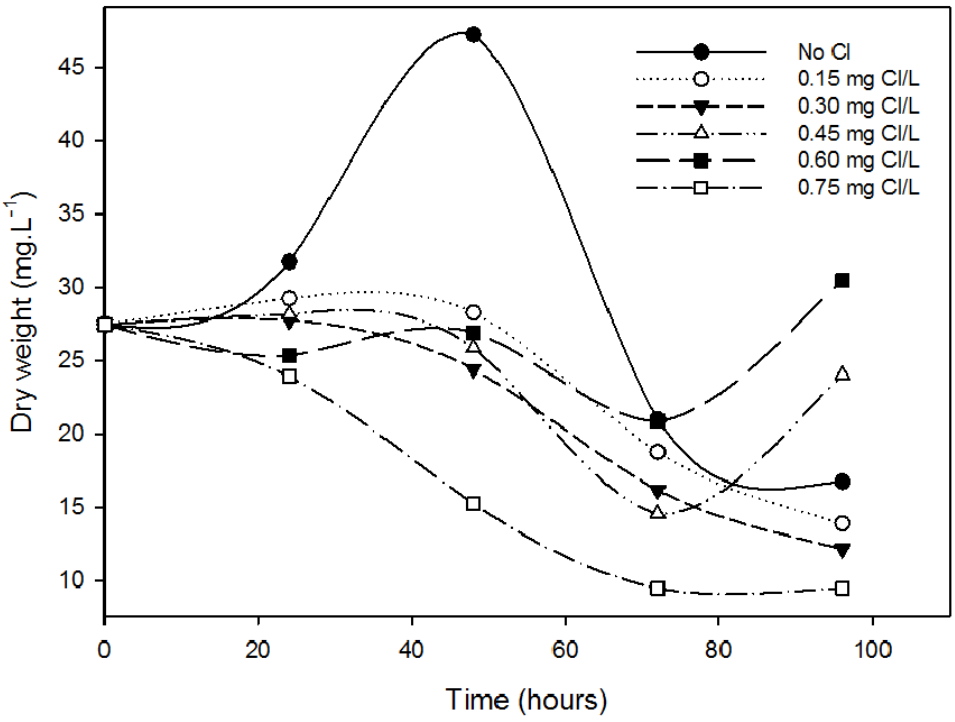


Figure 11. Growth of *C. kessleri* (28 mg/L dry weight of *C. kessleri* and 6-hrs interval chlorine dosing)

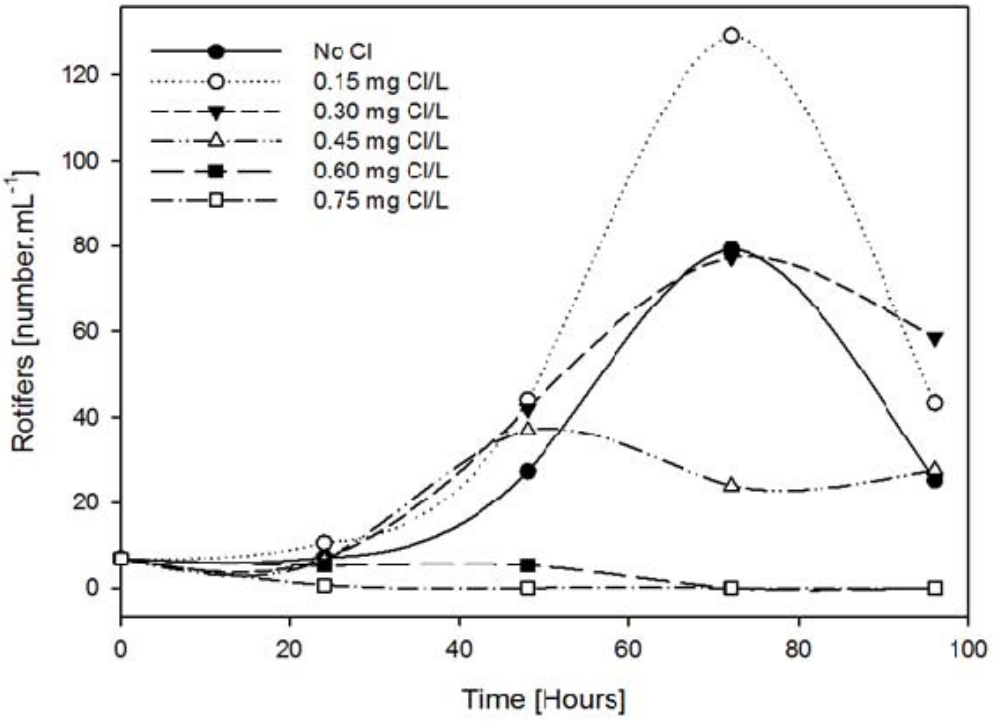


Figure 12. Variations in *B. calyciflorus* concentration for 96 hour duration (28 mg/L dry weight of *C. kessleri*, 6-hrs interval chlorine dosing and 7 neonates/mL concentration of *B. calyciflorus*)

A concentration of only 7 neonates/mL of *B. calyciflorus* was added to the control group. The dry weight of *C. kessleri* increased from 28 to 47.2 mg/L until 48 hours in the control group while the variant spiked concentration of chlorine ceased growth of *C. kessleri*. These control group decreased in algal concentration until an eventual crash. Chlorine dosing was stopped at 72 hours at all concentration ranges because population of *B. calyciflorus* on 0.60 and 0.75 mg Cl/L was significantly inhibited at 72 hours. In the case of 0.45 mg/L of chlorine intermittently spiked every 6 hours, rotifers were moderately inhibited from 72 hours and maintained similar population levels up to 96

hours.

After 72 hours, the growth rate of *C. kessleri* subjected to intermittent spiked concentrations of 0.45 and 0.60 mg Cl/L chlorine, respectively, nearly returned to its normal growth rate. Pond crashing could not be prevented by the 6-h intermittent chlorine dosing level below 0.30 mg Cl/L and above 0.75 mg Cl/L.

3.2.5.2 The Second Experiment for Toxicity of Chlorine on *C. kessleri* and *B. calyciflorus*

As defined in the chlorine dissipation test results section 3.2.1, chlorine was dissipated quite quickly in algal suspensions within 1~2 hours. Moreover, rotifers were observed to be groggy the first time they were exposed to chlorine above the 0.45 mg/L concentration, but looked very active again at the next 6 hours. Therefore, in order to maintain peak concentrations of chlorine for a long time, the chlorine dosing interval of the subsequent 2 experiments was reduced to 2 hours, which is the time approximately 90% of the chlorine residuals dissipated. In order to verify the growth rate results of the first experiment, that the growth rate of *C. kessleri* subjected to chlorine spikes at concentrations of 0.45 mg Cl/L and 0.60 mg Cl/L returned to a normal growth rate from 72 hours, the effects of these concentrations on the algal growth rate and rotifer population were observed by over 192 hours.

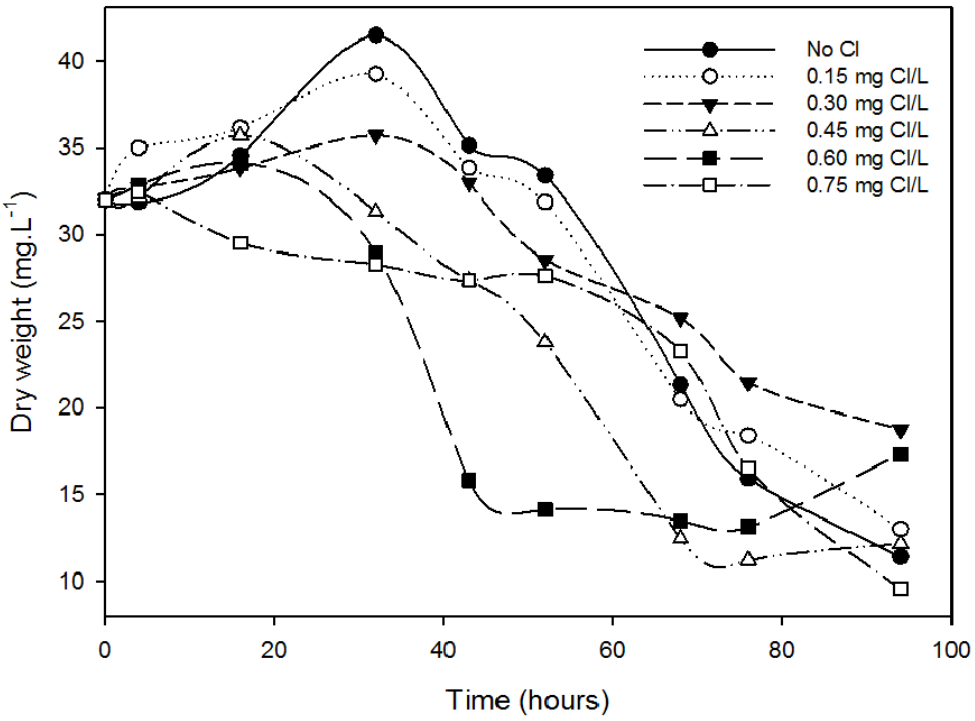


Figure 13. Growth of *C. kessleri* (32 mg/L dry weight of *C. kessleri* and 2-hrs interval chlorine dosing)

The results of the second experiment of chlorine toxicity on *C. kessleri* and *B. calyciflorus* for a 96 hour duration are shown in Figures 13 and 14. No *B. calyciflorus* was observed in 0.60 mg Cl/L and 0.75 mg Cl/L at 16 hours. Therefore, chlorine dosing was stopped at 16 hours to both these groups. Also, chlorine dosing to 0.45 mg Cl/L group was stopped at 32 hours due to significant inhibition of *B. calyciflorus*. All treatment groups of *C. kessleri* including the control group seemed to be proceeding towards a pond crash up to 76 hours. However, the dry weight of *C. kessleri* spiked with 0.45 mg Cl/L and 0.60 mg Cl/L gradually increased from 76 hours while the 0.15, 0.30 and 0.75 mg Cl/L levels of *C. kessleri* were heading towards a crash steadily. These did

not recover.

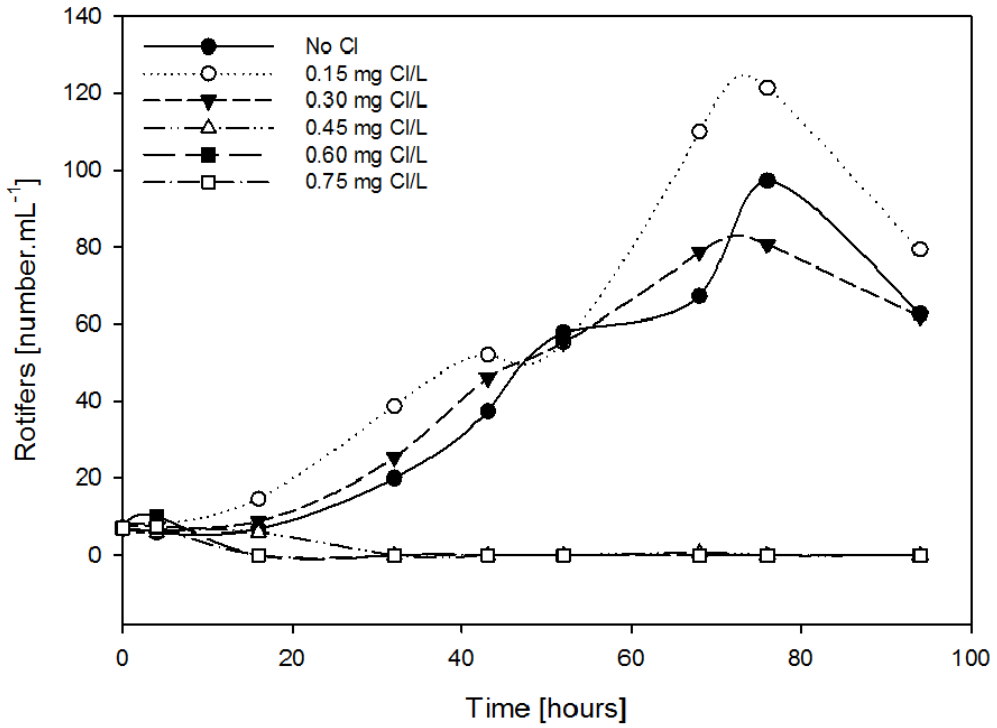


Figure 14. Variations in *B. calyciflorus* concentration for 96 hour duration (32 mg/L dry weight of *C. kessleri*, 2-hrs interval chlorine dosing and 7 neonates/mL of *B. calyciflorus*)

The dry weight of *C. kessleri* intermittently spiked with 0.45, 0.60 and 0.75 mg Cl/L every 2 hours was measured for 192 hours to track *C. kessleri* growth rate and population variations of *B. calyciflorus* after 96 hours. Figures 15 and 16 show the growth rate of *C. kessleri* and the population variations of *B. calyciflorus* up to 192 hours. Figure 15 clearly shows that *C. kessleri* spiked with 0.45 and 0.60 mg Cl/L recovered from 76 hours in the absence of its predation.

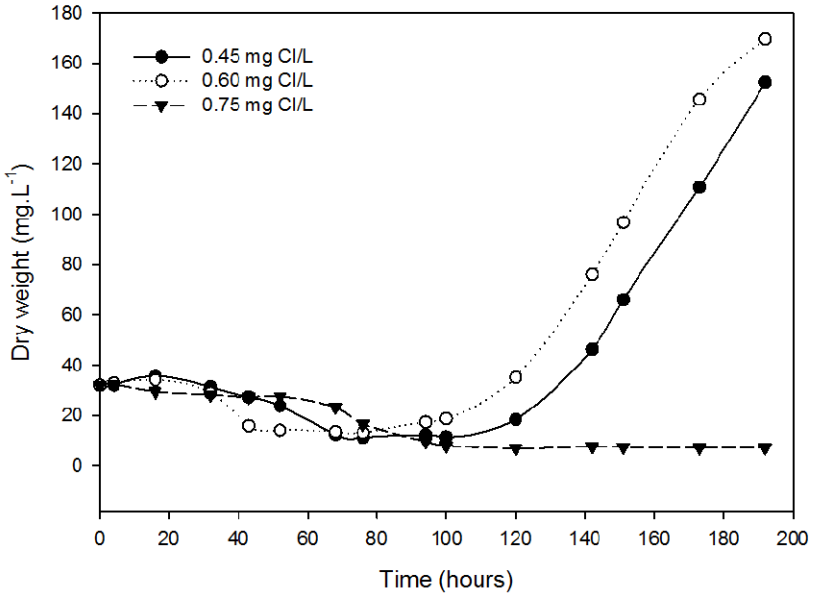


Figure 15. Growth of *C. kessleri* for 192-hrs in the second experiment

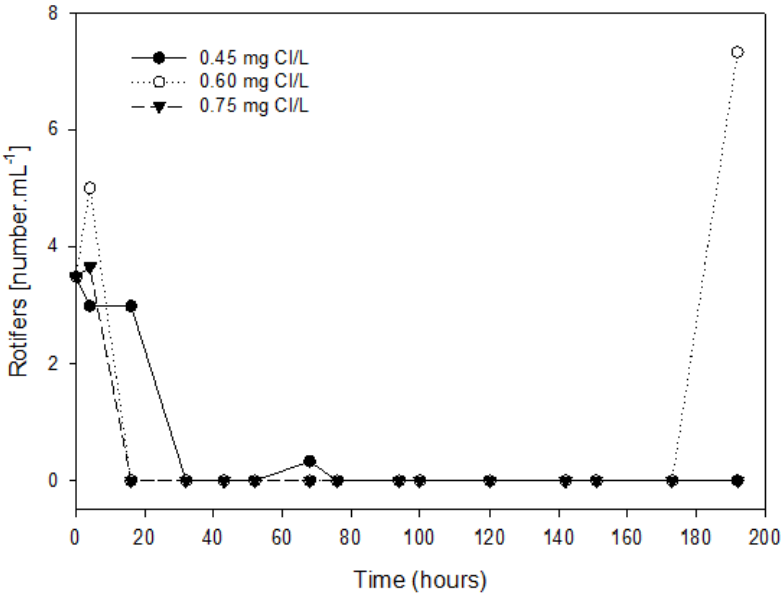


Figure 16. Variations in *B. calyciflorus* concentration for 192-hrs in the second experiment

After 76 hours, the specific growth rate of the recovered *C. kessleri* exposed to 0.45 and 0.60 mg/L intermittent chlorine was quite close to the normal algal growth rate. Figure 17 presents the normal growth rate of *C. kessleri* compared to two recovered *C. kessleri* growth rates after 76 h for 5-days.

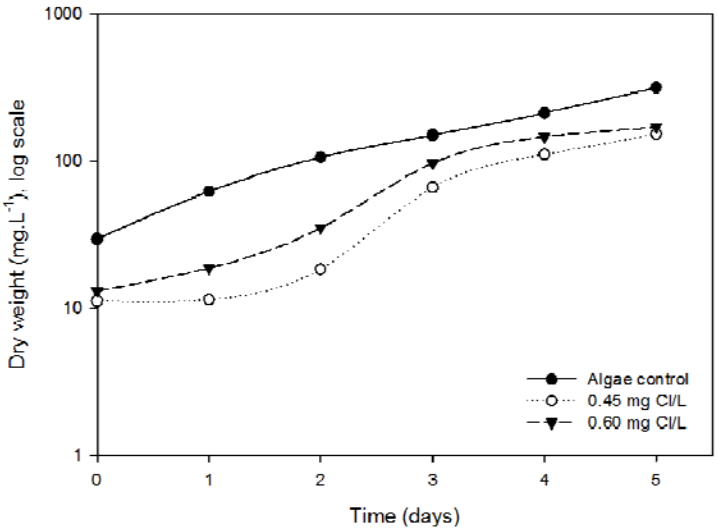


Figure 17. Growth rates of *C. kessleri* for 5-days (Control group, Treated groups spiked with 0.45 mg Cl/L and 0.60 mg Cl/L)

Specific growth rate (μ) of these three *C. kessleri* groups was calculated as

$$\mu = \ln\left(\frac{DW2}{DW1}\right)/(t2 - t1) \quad (1)$$

where, DW1 and DW2 are dry weight of biomass at time1 (t1) and time2 (t2), respectively (Levasseur, Thompson et al. 1993). Doubling time and divisions per day can be calculated using the equation below.

$$Divisions\ per\ day = \mu/ln2 \quad (2)$$

$$Doubling\ time = \frac{1}{Divisions\ per\ day} \quad (3)$$

The results of three growth rates of *C. kessleri* present in Table 3.

Table 3. Doubling time of *C. kessleri* control group and *C. kessleri* treated groups by chlorine, in the second experiment, 5-days duration

	C. kessleri control	*C. kessleri* exposed to 0.45 mg Cl/L	*C. kessleri* exposed to 0.60 mg Cl/L
Specific Growth rate	0.47	0.52	0.51
Divisions per day	0.683	0.753	0.738
Doubling time (days)	1.464	1.327	1.354

According to these results, the growth of *C. kessleri* intermittently spiked with 0.45 to 0.60 mg Cl/L every 2 hours, was not affected by chlorine if chlorine dosing was stopped and then *C. kessleri* was recovered when its predators were significantly inhibited.

3.2.5.3 The Third Experiment for Toxicity of Chlorine on *C. kessleri* and *B. calyciflorus*

The previous two experiments of toxicity of chlorine on *C. kessleri* and *B. calyciflorus* show how different chlorine dosing intervals affect inhibition of *B. calyciflorus* and growth of *C. kessleri*. The effective concentration of chlorine in two cases was between 0.45 mg/L and 0.60 mg/L while a pond crash cannot be avoided with *C. kessleri* algal suspension exposed to spiked chlorine under 0.3 mg Cl/L due to the weak toxicity on *B. calyciflorus*. Furthermore, *C. kessleri* exposed to above 0.75 mg Cl/L never recovered despite the fact that chlorine was not spiked after predator presence was

no longer observed.

One very important factor in finding the effective concentration of chlorine to inhibit rotifers is the initial algal biomass concentration necessary to survive this chlorine concentration (Trotter, Hendricks et al. 1978). In this respect, the concentration of *C. kessleri* in the third experiment was initially 57 mg/L, approximately double that of the previous two experiments. All other testing conditions were the same as the second experiment above except the initial algal suspension concentration. Figures 18 and 19 show the dry weight of *C. kessleri* and *B. calyciflorus* concentrations for 120 hr.

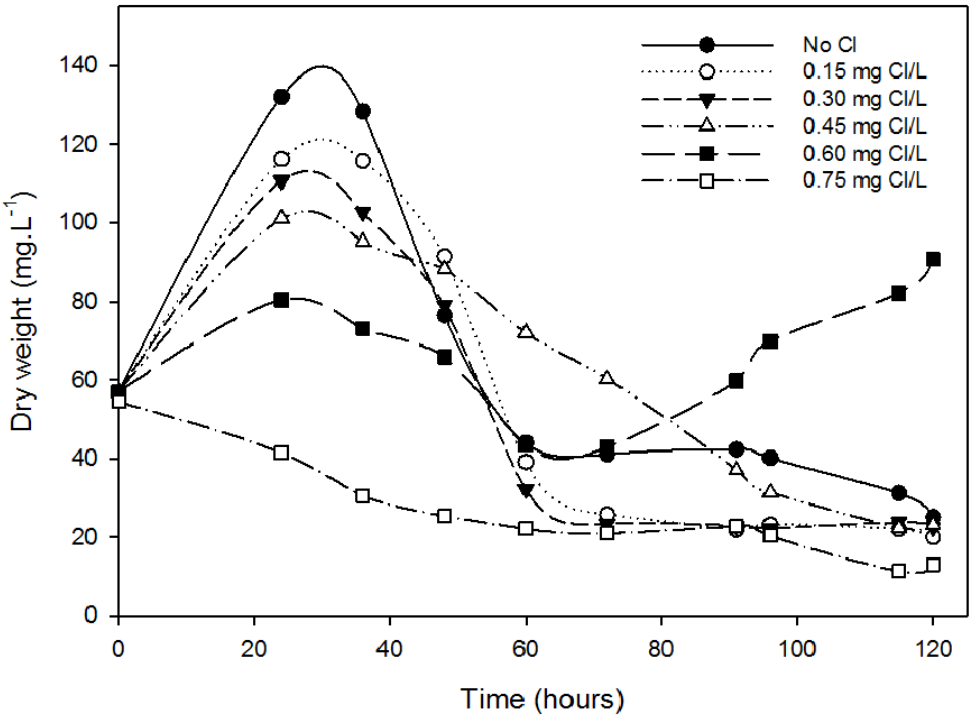


Figure 18. Growth of *C. kessleri* (57 mg/L dry weight of *C. kessleri* and 2-hrs interval chlorine dosing)

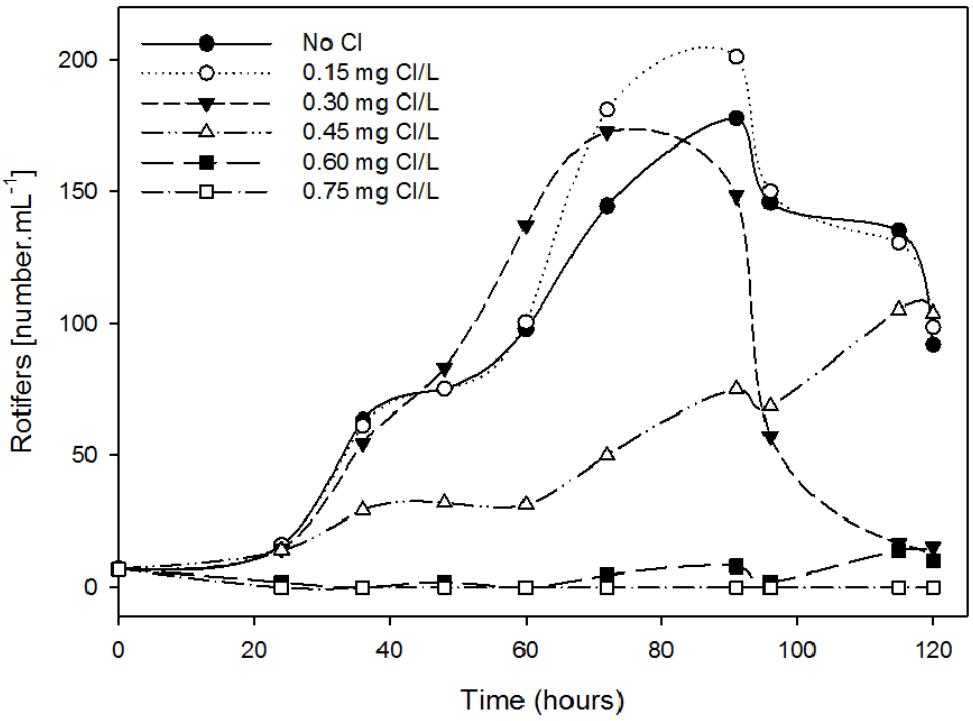


Figure 19. *B. calyciflorus* concentration for 120 hours (57 mg/L dry weight of *C. kessleri*, 2-hrs interval chlorine dosing and 7 neonates/mL concentration of *B. calyciflorus*)

In this experiment, *B. calyciflorus* was significantly inhibited in chlorine concentrations of 0.60 and 0.75 mg Cl/L at 36 hours. Thus, intermittent chlorine dosing to those two groups was stopped at 36 hours to observe if *C. kessleri* could recover from this point in time. All treatment groups of *C. kessleri* including the control group were also proceeding towards a pond crash up to 60 hours. However, only the *C. kessleri* spiked with 0.60 mg/L of chlorine survived and continued to grow gradually as of 72 hours while *C. kessleri* spiked with 0.15, 0.30, 0.45 and 0.75 mg Cl/L did not recover.

Unlike the previous experiment, *B. calyciflorus* spiked with 0.45 mg/L of chlorine was not significantly inhibited in this test. Those were inhibited by 68% (compared to

control group, no Cl), and maintained the same level up to 60 hours. Chlorine dosing to 0.45 mg Cl/L group was stopped at 60 hours since the dry weight of *C. kessleri* spiked with 0.45 mg/L chlorine had been reduced by 36% from its peak dry weight value. *B. calyciflorus* concentration in 0.45 mg Cl/L increased from this point, eventually increasing to the same level as the control group. When chlorine was spiked into 0.45 mg/L again from 91 to 101 hours, the population density of *B. calyciflorus* decreased for a short time, then continuously increased after 101 hours while the dry weight never increased.

Only the dry weight of *C. kessleri* intermittently spiked with 0.60 mg/L of chlorine was recorded for 186 hours in order to track the growth rate of *C. kessleri* and *B. calyciflorus* concentrations, as shown in Figure 20.

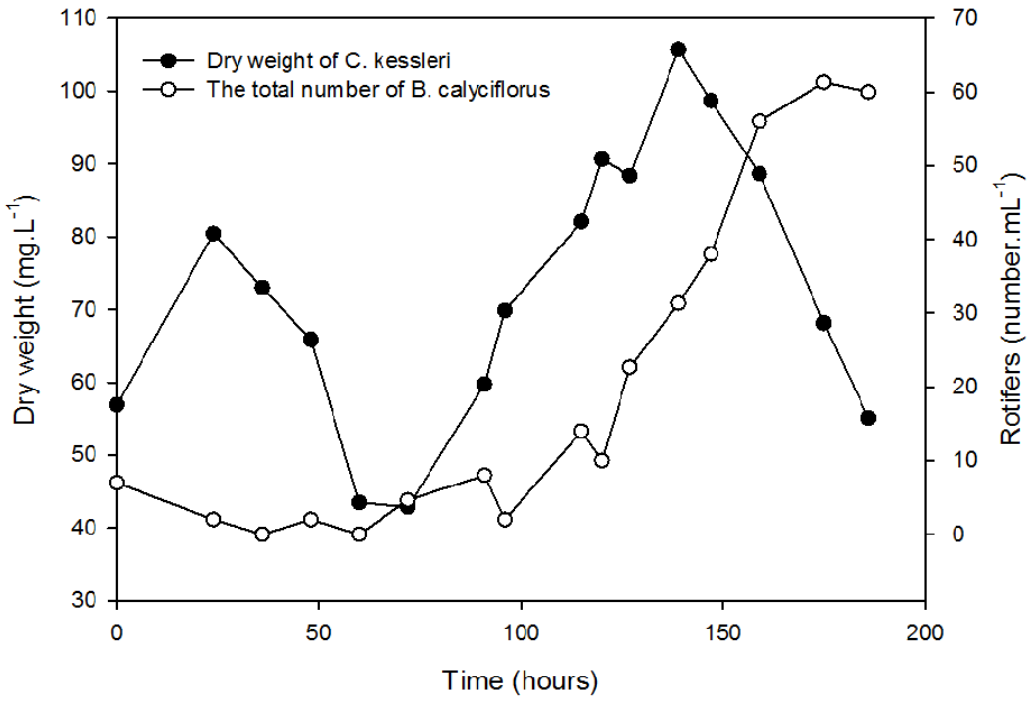


Figure 20. Growth of *C. kessleri* and B. calyciflorus concentrations for 186-hrs in the third experiment, 0.60 mg Cl/L only

Proceeding in a like manner as the previous experiment, *B. calyciflorus* was significantly inhibited by 72 hours. As well, *C. kessleri* was concurrently highly inhibited due to oxidation with chlorine. The dry weight of *C. kessleri* highly increased as of 72 hours and it reached 106 mg/L of dry weight at 139 hours. However, *C. kessleri* could not survive at 186 hrs due to the highly increased concentration of *B. calyciflorus* concentration of 61 rotifers/mL without chlorine dosing.

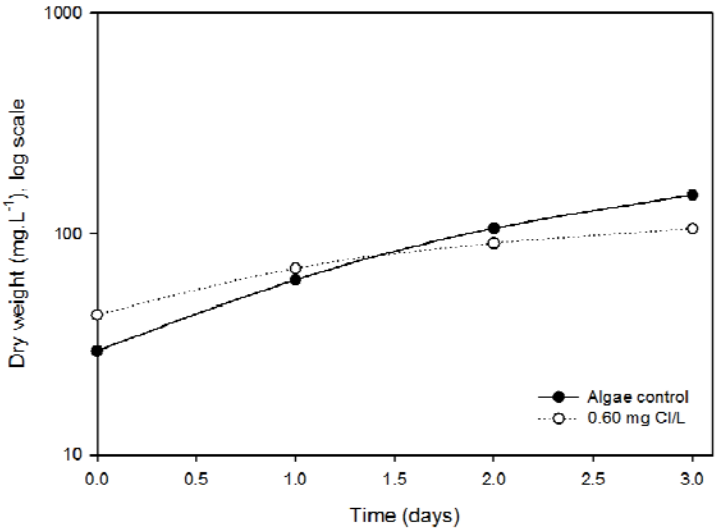


Figure 21. Growth rates of *C. kessleri* for 3-days (Control group and Treated group spiked with 0.60 mg Cl/L)

Despite the fact that *C. kessleri* exposed to 0.60 mg Cl/L survived and continued to grow from 72 h to 139h, its specific growth rate was very low compared to the normal growth rate of *C. kessleri* under this test condition. (Table 4)

Table 4. Doubling time of *C. kessleri* control group and *C. kessleri* treated group by chlorine, in the third experiment for 3-days

	C. kessleri control	*C. kessleri* exposed to 0.60 mg Cl/L
Specific Growth rate	0.54	0.30
Divisions per day	0.782	0.434
Doubling time (days)	1.280	2.305

C. kessleri concentration fluctuates with the population of *B. calyciflorus*. Once *B. calyciflorus* was significantly inhibited by 0.6 mg Cl/L intermittent dosing at 36 hours, the dry weight of *C. kessleri* was highly increased. Then, as *B. calyciflorus* concentrations increased, conversely, the dry weight of *C. kessleri* decreased. The difference with the second experiment is the resilience of *B. calyciflorus* by chlorine. In the second experiment, *B. calyciflorus* spiked with 0.45 mg Cl/L did not recover from 32 hours to 173 hours, and *B. calyciflorus* concentrations spiked with 0.60 mg Cl/L never recovered from 16 hours to the test duration of 192 hours. However, *B. calyciflorus* concentrations spiked with 0.60 mg Cl/L in the third experiment were not much more inhibited than those of the second experiment. *B. calyciflorus* was observed even within 100 hours, and increased highly from the time of cessation of chlorine dosing.

According to these results, it is readily assumable that higher algal biomass concentration is needed in the presence of higher concentrations of chlorine to inhibit predation. The higher concentration of organic matter can trigger oxidation by chlorine and lead to further reduction of chlorine toxicity. In the case of the 0.75 mg/L chlorine

dosing level, despite all *B. calyciflorus* being eliminated within 24 hours, *C. kessleri* could not survive the test duration due to the high toxicity of chlorine to algae.

4. CONCLUSION AND RECOMMENDATIONS

4.1 Conclusion

The overall goal for this research was to find the concentration of the chlorine that will significantly inhibit rotifers, *Brachionus calyciflorus*, without significantly reducing algae growth, *Chlorella kessleri*. Chlorine dissipation test, acute toxicity test of chlorine to rotifers, chlorine toxicity test for algae, pond crash test and chlorine toxicity test for rotifer and algae combined environment were conducted to investigate the characteristics of the test rotifer and algae, especially those where they exist together in algal suspension. Based on the results of these experiments, the following conclusions can be reached:

I. Half of mortality of test rotifers, *B. calyciflorus*, in spring water was investigated through acute toxicity test and the 24-hr LC_{50} was 0.198 mg Cl/L.

II. Half of mortality of the freshwater algae, *C. kessleri*, was investigated and the 24-hr LC_{50} was 0.321 mg Cl/L. Based on these two tests, it was determined that *Brachionus calyciflorus* was more sensitive to chlorine than *Chlorella Kessleri* and chlorine may be used to avoid algae pond crashes by *B. calyciflorus.*

III. However, the chlorine dissipation rate in algal suspension was quite different from that of distilled or spring water. In the chlorine dissipation test, chlorine residual dissipated 70 and 88% within the first hour and 85 and 90% by the second hour at an algae concentration of 9.1 and 183.6 mg/L, respectively. Based on this finding, chlorine dosing interval was determined as 6 hours and 2 hours for the chlorine toxicity tests on *B. calyciflorus* and *C. kessleri* in a combined environment.

IV. The pond crash rate of *C. kessleri* by three different *B. calyciflorus* concentrations was investigated. The dry weight of *C. kessleri* with below 7 neonates/mL *B. calyciflorus* concentration was increased by max. 85% of control algae growth. This lead to a pond crash, while *C. kessleri* containing over 14 neonates/mL *B. calyciflorus* did not grow at all during the test period. Also, the reproduction rate of *B. calyciflorus* was examined. Rotifer concentrations did not increase in the first 24 hours, however it was highly proliferated after the log phase of 24-hrs. According to the pond crash rates by *B. calyciflorus*, rotifer concentration was determined to be 7 neonates/mL for the chlorine toxicity test on *B. calyciflorus* and *C. kessleri* in a combined environment.

V. The toxicity test of chlorine on *B. calyciflorus* and *C. kessleri* in a combined environment consisted of three experiments as shown below:

- ✓ Dry weight of *C. kessleri*: 28 mg/L, Chlorine dosing interval: 6 hrs.
- ✓ Holding dry weight of *C. kessleri* as 32 mg/L, Chlorine dosing interval: 2 hrs.
- ✓ Dry weight of *C. kessleri*: 57 mg/L, Holding Chlorine dosing interval at 2 hrs.

B. calyciflorus concentration was 7 neonates/mL for all experiments. Effective intermittent concentration of chlorine to significantly inhibit rotifers when *B. calyciflorus* exist together with *C. kessleri* culture was from 0.45 to 0.60 mg Cl/L, and a short period of chlorine dosing intervals was effective. Decreasing of algae concentration was unable to be prevented due to oxidation between algal compounds and chlorine. However, *C. kessleri* spiked with 0.45 to

0.60 mg Cl/L returned to a normal growth rate after predation was significantly inhibited.

The summary of the results of these experiments are shown below:

Table 5. The results summary of the toxicity of chlorine on *B. calyciflorus* and *C. kessleri*

	Cl concentration of survived *C. kessleri*	Inhibition level, The time to inhibit *B. calyciflorus*	The time of *B. calyciflorus* repopulation
28 mg/L *C. kessleri* + 6 hrs interval Cl dosing	0.45 mg Cl/L	Moderately, 48 hrs	Maintained at moderate level
	0.60 mg Cl/L	Significantly, 72 hrs	Never reemergence
32 mg/L *C. kessleri* + 2 hrs interval Cl dosing	0.45 mg Cl/L	Significantly, 32 hrs	After 200 hrs
	0.60 mg Cl/L	Significantly, 16 hrs	192 hrs
57 mg/L *C. kessleri* + 2 hrs interval Cl dosing	0.60 mg Cl/L	Significantly, 36 hrs	72 hrs

4.2 Recommendations

This research examined the toxicity of chlorine on rotifers in algal suspension concentration from 28 to 57 mg/L of dry weight. Chlorine concentrations of 0.45 and 0.60 mg Cl/L showed significant inhibition to rotifers in 28 and 32 mg/L dry weights of algal biomass, however 0.45 mg Cl/L did not have an effect on 57 mg/L dry weight of

algal biomass. Thus, the relationship between algal biomass concentration and effective intermittent chlorine concentration may need to be studied further.

The results of this research may be applied to control rotifer quantities when an algae culture (especially, open pond) has been contaminated by rotifers. For example, if rotifers are detected in an open pond culturing system, 0.60 mg Cl/L of intermittent chlorine can be spiked into a well-mixed pond. Rotifers are then significantly inhibited, and chlorine dosing should be stopped after control of the rotifer population. Based on this concept, future research will investigate whether appropriate concentrations of chlorine automatically depend on the rotifer population. Furthermore, future research will aim to find other chemicals (or synthetic chemical) that can effectively inhibit rotifers without having an ill effect on the algal commercial crop.

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