AMERICAN UNIVERSITY OF BEIRUT

MICROCYSTIN RELEASE FOLLOWING THE CHLORINATION OF HYDROGEN PEROXIDE TREATED MICROCYSTIS-LADEN WATERS

by
AYDA MOHAMAD NAWAM

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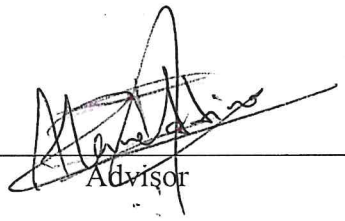

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AN ABSTRACT OF THE THESIS OF

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Title: Microcystin release following the chlorination of hydrogen peroxide treated microcystis-laden waters

Freshwater systems worldwide are facing increased risks of impairment as a result of the proliferation of harmful algal blooms. Moreover, many cyanobacterial blooms release toxic compounds that are referred to as cyanotoxins. One of the most problematic bloom forming cyanobacteria resulting from anthropogenic-induced eutrophication is *Microcystis aeruginosa*, which is capable of releasing Microcystin. While most of its cyanotoxins are intracellular, they can become available to the environment when the cells lyse. Conventional water treatment processes are unable to remove these toxins that have been found in finished water. In an effort to control these blooms, chemical algaecides are typically applied. In this study, the efficacy of two algaecides, namely hydrogen peroxide (H_2O_2) and copper sulphate ($CuSO_4$), was first assessed with regards to their abilities to reduce *Microcystis* levels and minimizing intra-cellular toxin leakage. The results showed that while applying high dosages of the two algaecides inhibited *Microcystis*, the total toxin levels tended to exceed the 1 µg/L World Health Organization (WHO) recommended standard. The impacts of chlorinating algaecide treated water were then quantified. The results showed that the use of $CuSO_4$ followed by a subsequent filtration and chlorination step proved to be the most effective approach towards inhibiting the cyanobacteria and reducing toxin levels. Yet, toxin and trihalomethane levels were found to be high when chlorination occurred within the first 48 hrs of algaecide application. Filtrating and chlorinating water that had been treated with H_2O_2, showed a significant drop in toxin levels; yet the WHO standard was not met irrespective of the H_2O_2 dose or when the chlorination was implemented.

Keywords: Algaecides, Hydrogen Peroxide, Copper Sulfate, Chlorine, *Microcystis aeruginosa*. Microcystin, MC-LR.

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CHAPTER 1

INTRODUCTION

Water eutrophication is a global environmental concern that is responsible for the impairment of ecosystems and deterioration of water quality in lakes and reservoirs. Nutrient loading into water bodies, especially phosphorous and nitrogen, is one of the main factors responsible for water eutrophication and cyanobacteria induced Harmful Algal Blooms (HABs) (Bartram & Chorus, 1999; Buratti et al., 2017; Carmichael, 1997; Conley et al., 2009; Elliott, 2010; Khan & Ansari, 2005; Paerl et al., 2016; Paerl & Scott, 2010b). Cyanobacteria, also known as blue-green algae, belong to the gram-negative bacteria and can be commonly found in water bodies such as surface freshwater, wastewater stabilization ponds, and marine ecosystems (Bartram & Chorus, 1999; Heisler et al., 2008; Ho et al., 2010; Rastogi, Sinha, & Incharoensakdi, 2014). Nevertheless, their blooms can be toxic and disruptive, leading to the impairment of many important freshwater systems worldwide such as Lake Taihu in China (Davis, Berry, Boyer, & Gobler, 2009; Paerl & Otten, 2013; Paerl & Scott, 2010a), Lake Erie in North America (Davis & Gobler, 2016), lake Okeechobee and Apopka in Florida (Beaver & Havens, 1996; Coveney, Stites, Lowe, Battoe, & Conrow, 2002), Lake Washington, as well as most of the shallow freshwater lakes in the western and northern regions of the Netherlands (Doblin, Coyne, Rinta-Kanto, Wilhelm, & Dobbs, 2007; Gulati & Van Donk, 2002; Michalak et al., 2013; Paerl & Otten, 2013; Paerl & Scott, 2010b). In Lebanon, the Qaraoun reservoir has suffered from toxic cyanobacterial blooms regularly, ever since they were first reported in 2009 (Atoui, Hafez, & Slim, 2013; Dia, Alameddine, & El-Fadel, 2019).

Several cyanobacteria genera are known to form blooms and produce cyanotoxins including *Microcystis, Anabaena, Nodularia, Cylindropermopsis, Aphanizomenon, Oscillatoria,*

and Planktothrix. Note that *Microcystis* blooms are by far the most common worldwide. Cyanotoxins pose a direct risk to humans and animals by causing skin irritations, liver cancer (hepatotoxic), and neurotoxicity (Bartram & Chorus, 1999; Buratti et al., 2017; Carmichael, 1992; Codd et al., 1999; Massey et al., 2018; Ueno et al., 1996; Zimba, Khoo, Gaunt, Brittain, & Carmichael, 2001). Micrcocystins (MCs) are one of the most commonly occurring hepatotoxins released by cyanobacteria. There are over 80 variants of MCs, of which the most commonly occurring and toxic is known as microcystin-leucine arginine (MC-LR) (Bartram & Chorus, 1999; Svrcek & Smith, 2004). While the MCs are produced and stored intracellularly, they can be released into the surrounding environment when the cell membranes are lysed either naturally or through physical or chemical stress (Li, Li, & Li, 2017; Q. Wang et al., 2010; Watanabe & Oishi, 1985). MC-LR are known to bio-accumulate in aquatic animals and get magnified as they move up the food-web. As such, MC-LR can have a direct risk on the human health (Chen, Chen, Zhang, & Xie, 2016; Lone, Koiri, & Bhide, 2015; Smith & Haney, 2006). MC-LR has been classified as a possible carcinogen by the International Agency for Research on Cancer (IARC, 2010) and as such, the World Health Organization has defined the 1 μg/L of total MC/LR in drinking water as a provisional guideline (Michalak et al., 2013; WHO, 2003). Water establishments worldwide are increasingly being asked to upgrade their systems in order to minimize the risk of exposure to these toxins in their treated waters.

Various mitigation measures have been proposed to control algal blooms, including nutrient control, physical, biological, and chemical strategies (Drabkova, 2007). While reducing nutrient inputs into water bodies, especially phosphorous and nitrogen, might be the most effective approach towards reducing the growth of cyanobacteria on the long run (Brookes & Carey, 2011; Conley et al., 2009; Jankowiak, Hattenrath-Lehmann, Kramer, Ladds, & Gobler,

2019), opting for such measures might not be feasible given the difficulty of implementing non-point source controls and the required time for the system to readjust, especially with nutrient buildup in the sediments. The use of mechanical mixing and/or biological manipulation has been found to be costly and/or ineffective as an immediate solution to HABs (Benndorf, Böing, Koop, & Neubauer, 2002; Visser, Ibelings, Van Der Veer, Koedood, & Mur, 1996). As such, the use of chemical control methods, such as the application of algaecides, remains the most commonly adopted mitigation measure given that they provide immediate effects.

Many water agencies have opted for the application of algaecides to control HABs in their impaired reservoirs and lakes (Fan, Hobson, Ho, Daly, & Brookes, 2014). Copper sulphate ($CuSO_4$), which has been in use since the early 1900's, remains the most popular algaecide largely due to its low cost, effectiveness, and ease of application (Dia, Alameddine, Salam, & El-Fadel, 2016; Elder & Horne, 1978; Hobson et al., 2012). Hydrogen peroxide (H_2O_2) is another algaecide that has been promoted as a substitute to copper sulphate (Matthijs et al., 2012) as it can be effective at controlling cyanobacterial cells and oxidizing their toxins (Svrcek & Smith, 2004). H_2O_2 is also considered to be more eco-friendly as compared to other algaecides (Barrington, Ghadouani, & Ivey, 2011). H_2O_2 acts on damaging and lysing the membrane integrity of cyanobacterial cells (Fan et al., 2014; Mikula, Zezulka, Jancula, & Marsalek, 2012; Qian et al., 2010) and in limiting their photosynthetic activity (Qian et al., 2010). Yet, the lysing of the cell membranes after the application of H_2O_2 treatment has been shown to release intracellular toxins. Several studies have reported that some of the released intracellular MC-LR gets oxidized by the remaining hydrogen peroxide in the water (Fan et al., 2014; Matthijs et al., 2012; Qian et al., 2010). Yet, the fast decay of H_2O_2 in the water can limit its ability to oxidize completely the released toxins (Cooper, 1994). The effectiveness of H_2O_2 has been tested under a

wide range of dosage from as high as 100 mg/L (Barrington et al., 2011) to 60 mg/L (Gao et al., 2015; Z. Wang, Li, Qin, & Li, 2012), down to to < 5 mg/L (Drabkova, 2007; Matthijs et al., 2012). Despite that fact that its algaecidal effectiveness increases with dose, opting for elevated dosages poses serious threats to the environment.

As more water agencies opt to apply algaecides to their water bodies in an effort to control HABs event, it is critical that the impacts of their indiscriminate use on the environment, on intracellular toxins release, and on the taste and odor of treated waters are better assessed (McElhiney & Lawton, 2005). Few water and environmental agencies have started to impose restrictions so as to limit their application to the early stages of a HAB event, when the concentrations of cells and their levels of toxins are still low (Newcombe, House, Ho, Baker, & Burch, 2010).

One of the concerns that water agencies are having to deal with is to ensure the safety of chlorinating algaecide treated waters. While chlorine has well established algaecidal effects on cyanobacteria, the impacts that chlorination has on algaecide-treated waters remains poorly studied. It has been shown that chlorine can cause further damage to *Microcystis* cells thus accelerating the release of toxins into the water (Daly, Ho, & Brookes, 2007). Yet, some studies have reported that the residual chlorine can effectively degrade the extracellular cyanobacterial toxins (Ho, Lambling, Bustamante, Duker, & Newcombe, 2011; Nicholson, Rositano, & Burch, 1994).

In this study, we first evaluate the efficacy of two commonly used algaecides ($CuSO_4$ and H_2O_2) on controlling *Microcystis aeruginosa* over 96 hrs, while assessing the levels of intra and extra-cellular MC-LR post each application with an emphasis on assessing cell integrity. The thesis then attempts to assess the combined impacts of chlorinating algaecide-treated waters,

with a focus on assessing the risks associated with increasing *Microcystis* cell lysing, the generation of trihalomethanes (THMs), and exposure to elevated MC-LR levels. Two different dosages of chlorine are examined, while the time of when the chlorine application happens post-algaecide use is explored. The study concludes by recommending an optimal window of time, when a safe dose of chlorine can be safely applied post-algaecide application.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cyanobacteria culturing

Water samples containing naturally occurring *Microcystis aeruginosa* cells were collected 10 cm below the water surface from the Qaraoun reservoir during mid-July 2019. The reservoir is well known to be hypereutrophic and experiences consistent *Microcystis aeruginosa* blooms (Atoui et al., 2013) throughout the summer months. The samples were transported on ice to the Environmental Engineering Research Center (EERC) laboratory in AUB. Microscopic observations of the bloom were immediately performed upon arrival to the lab and the presence of *Microcystis aeruginosa* was confirmed. Additionally, the observations confirmed that *Microcystis aeruginosa* were predominately colonial. Chlorophyll-a levels, cell-densities, nutrients, pH, and temperature were also measured post sample collection. pH and temperature levels that were measured on site. Samples were then cultivated in 20 L glass flasks enriched with BG 11 medium (Sigma Aldrich, BG-11 Freshwater Solution, 50X). Samples were cultured under a 12:12 light:dark cycle. Air pumps were operated in all flasks to provide aeration, enrich mixing, and ensure no carbon limitation. The flasks were placed in a plexiglass enclosure and their temperature was continuously monitored and maintained at 25°C (USEPA, 2002) by operating a fresh-air fan that automatically turned on whenever the temperature increased above 25 ^{o}C.

The algae were harvested in their exponential growth phase after high cell densities (up to $1x10^{8}$ cells/ml) were achieved. The desired cell density was reached in around 10 days. Cell density was determined by using a hemocytometer under a Zeiss Fluorescence microscope (Axiovert

200). Harvested cyanobacteria were then sub-cultured in 500 ml Erlenmeyer flasks and diluted with Milli-Q water and fortified with BG-11 to reach a final volume of 500 ml, have ~ $1x10^7$ cells/ml, and maintain the BG-11 standard recipe. A total of 31 flasks were prepared and used for the algaecide inhibition experiments.

2.2 Experimental procedure

2.2.1 Inhibition tests

The inhibition tests were conducted in the 500 ml Erlenmeyer flasks with a starting cell density of $1x10^7$ cells/ml in 500 ml (Figure 1). Each flask was then exposed to one of a set of predefined concentrations of the $CuSO_4$ and H_2O_2 algaecides. Stock solutions of each of the two algaecides were used to reach the required dosages. H_2O_2 dosages were prepared using Sigma Aldrich H-1009 30 % w/w solution, while the $CuSO_4$ dosage was prepared from Sigma Aldrich Copper (II) sulfate pentahydrate 209198 ACS reagents. Accordingly, different volumes of the stock solution of H_2O_2 were added to reach the three final dosages of hydrogen peroxide that were tested namely, the 1, 7, and 10 mg/L. On the other hand, only the 1 mg/L $CuSO_4$ dose was prepared from the stock solution. The latter was used to bench-mark against the algaecidal efficiency of the three dosages of hydrogen peroxide, given that the 1 mg/L $CuSO_4$ dose is a typical dose used in algae control.

All inhibition experiments were conducted according to the EPA method (Lewis et al., 1994). Inhibition flasks were placed within an incubation cabinet and irradiated with florescent lamps providing 4306 LUX (Lewis et al., 1994). Mixing of the samples was performed using magnetic agitators. Triplicate inhibition tests were conducted for each of algaecide-dose combination. Moreover, three control flasks were prepared and monitored. Post-algaecide application, water samples from each flask were taken periodically every 24 hours over a 4 day period to undergo

analysis. The daily analysis included measuring temperature, pH, Chl-a levels, cell counts, cell lysing through flow cytometer, MC-LR concentrations, total organic carbon (TOC), dissolved organic carbon (DOC), and THM. Temperature and pH were measured using a HACH SensION+ MM110 portable probe.

Figure 1 Samples added to 500 ml Erlenmeyer flaks, labeled, and mixed by magnetic stirrer

Chlorophyll-a concentrations were measured by filtering 50 mL samples through glass microfiber filter papers (Whatman 47 mm GF/C). The filter papers were then sonicated in 3 mL of boiled ethanol solution (90%). Extracts (filter papers together with ethanol) were seeped in 10 mL 90% ethanol solution overnight to be later clarified using centrifugation (15 mins at 3000-5000 G). Chlorophyll-a concentrations were calculated based on absorbance (Standard Method 10200 (HS2)) (American Public Health, Eaton, American Water Works, & Water Environment, 2005; Rice, Baird, Eaton, & Clesceri, 2012) using a HACH DR 3900 spectrophotometer. The chlorophyll-a concentration was calculated as follow:

$$Chlorophyll\ a = \frac{29.62 * (665a - 665b) * V_e}{V_s * L}$$

where: V_e: Volume of ethanol extract (mL)

V_s: Volume of water sample (L)

L: Path length of vial (cm)

665a: corrected 665a absorbance = 665-750

665b: corrected 665b absorbance after adding 0.01 ml of 1 mol/L HCl = 665-750

In addition to the chl-a measurements, the cells count of *Microcystis aeruginosa* were conducted microscopically using a Zeiss Fluorescence microscope (Axiovert 200) fitted with a hemocytometer. As *Microcystis* can exist either in unicellular or colonial form, a separation technique was necessary to achieve an accurate cell count. Breaking up colonies can be achieved through several methods including boiling, alkaline hydrolysis (80-90^{o}C for 15 mins, followed by intensive mixing), and sonication (Bartram & Chorus, 1999; Humphries & Widjaja, 1979). Alkaline hydrolysis was found to be time consuming, while prolonged sonication had the potential of rupturing the cells. In this study, heat treatment was used to break up the *Microcystis* colonies. This method resulted in the complete separation of the cells and didn't lyse them. Samples were thus boiled for 5-10 mins to ensure that the colonies broke. Microscopic images were taken by a Canon Camera that was fitted to the microscope before uploading them into a calibrated automated cell counter software. The cell concentrations were calculated as follows:

$$Cell\ density\ (\frac{cells}{ml}) = \frac{Average\ of\ cells\ from\ 8\ squares * Dilution\ factor}{Volume\ of\ a\ square\ (ml)}$$

Cell integrity and the colonial status of the *Microcystis* were examined using flow cytometry in combination with fluorescent probes. A BD FACSAria flow cytometry equipped with both an octagon laser excited by the 488 nm (blue) as well as a trigon laser excited by the 633 nm (red)

was used. Light scatter measured through the FSC and SSC were used to assess the *Microcystis* population with regards to its breakdown between colonies and unicellular cells. Note that the FSC and SSC voltages were adjusted to better gate the population of interest. Phycocyanin autofluorescence was detected using the APC-H fluorochrome at 630 nm (also known as FL4/red laser) (Figure 2). Fluorescent signals were used to distinguish between healthy and dead *Microcystis* cells. A healthy population of *Microcystis* is expected to fluoresce when excited by the red laser. Moreover, the SYTOX green fluorescent dye (supplied by Molecular probes, Cat No. S-7020) was used to distinguish dead cells (Figure 3). SYTOX green is a membrane resistant fluorescent dye that cannot cross the intracellular membrane of a non-damaged cell; yet it can easily penetrate damaged cell membrane and bind to nucleic acids. As recommended, SYTOX Green was diluted in dimethyl sulfoxide (DMSO) to a stock concentration of 100 μM and stored in darkness at -20°C and then further diluted to 50 μM with deionized water before being applied to the samples (Daly et al., 2007; Regel, Brookes, Ganf, & Griffiths, 2004). For the measurement of *Microcystis* cell membrane integrity, 0.02 ml of the working solution of SYTOX Green was added to a 0.98 ml subsample of *Microcystis aeruginosa* in a Falcon polyester tube (12 x 75 mm) to achieve a 1 μM final concentration. The samples were then stained for 7 min in darkness at room temperature prior to flow cytometer analysis. Cells with higher fluorescence are those that have lost the integrity of the cell wall and thus allowed solutes into the cell. SYTOX green fluorescence was detected using FITC fluorochrome at 530 nm (also known as FL1/blue laser). Untimely gates were defined to separate the contents of the water into 4 groups namely, healthy unicellular cells, healthy colonies with some dead unicellular cells, dead cells and colonies, and background material in the water (Figure 4). The first gate was defined at 10^4 APC-H (voltage=414). Cells below that value were defined as dead cells or background material as they

did not show a high florescence, which indicates lack of phycocyanin. Another gate at 10^4 FITC-H (voltage=308) was also selected. Readings above that level indicated cells with high membrane damage thus reflecting the presence of dead cells, whereas cells below that level indicated low cell damage and as such reflecting the presence of healthy unicellular or colonial *Microcystis aeruginosa* population. These gates were defined by passing samples of healthy cells and comparing them to those that were subjected to boiling for 3 minutes to ensure that all cells were dead.

The two gates resulted in 4 quadrants (Figure 4). Readings in quadrant 1, were defined as dead *Microcystis aeruginosa* since they had high readings of FITC-H and low readings of APC-H. Readings in quadrant 2, were associated with healthy colonial cells with some damaged cells. These were associated with high fluorescence for both APC-H and FITC-H. Quadrant 3 (low FITC-H and APC-H) were assumed to contain background non-*Microcystis aeruginosa* particles. Readings in quadrant 4, were associated with healthy unicellular cells, as these were associated with high fluorescence in APC-H while FITC-H was low. The contents in quadrant 3 were removed from further analysis and the percentage of the remaining populations in the three quadrants were reassessed accordingly.

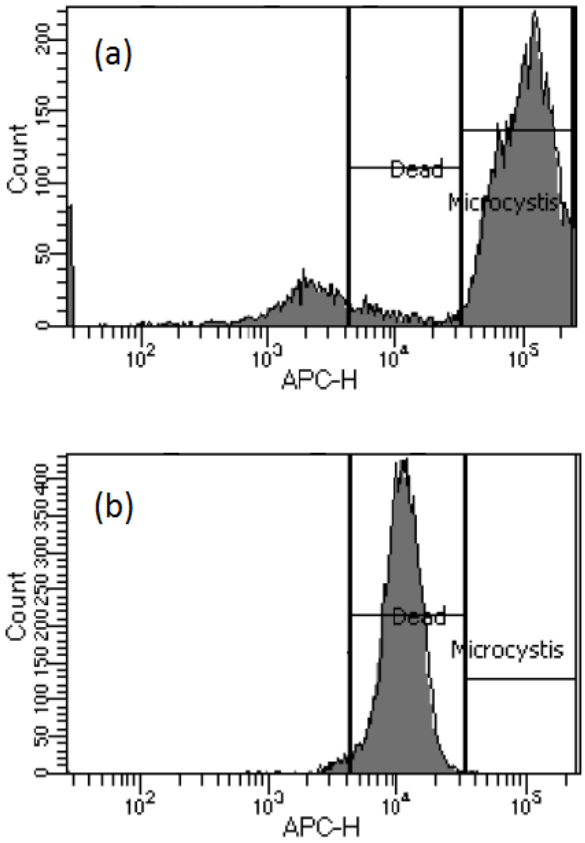


Figure 2 Gating regions representing (a) intact cells and (b) boiled damaged cells. Intact cells show high levels of APC-H as compared to damaged cells.

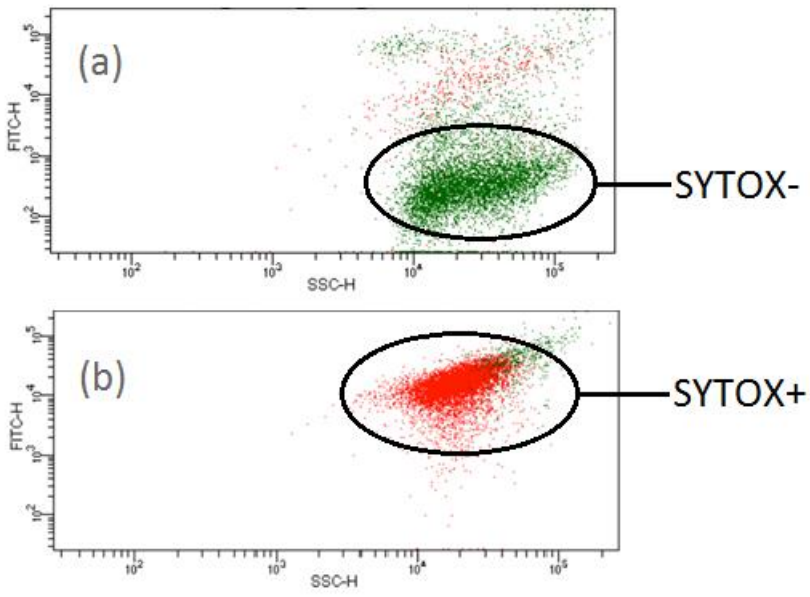


Figure 3 Gating regions representing (a) intact cells and (b) boiled damaged cells. Both samples were exposed to SYTOX green dye before reading. Damaged cells show high levels of FITC-H as compared to healthy cells.

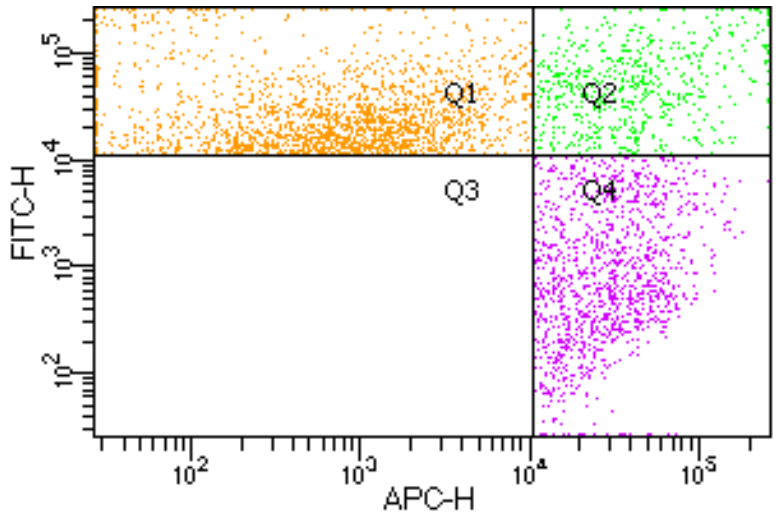


Figure 4 Flow cytometry results for a control sample of *Microcystis aeruginosa* showing APC-H vs. FITC-H corresponding to phycocyanin auto-fluorescence and SYTOX green dye. Q1 region represents dead cells, Q2 region represents healthy colonies with some dead unicellular cells, Q3 region represents background organic material, and Q4 region represents healthy unicellular cells.

Microcystin concentrations were determined using the Enzyme Linked Immunosorbent Assay (ELISA) technique (Lürling, Meng, & Faassen, 2014) that is approved by the USEPA (USEPA Method 546). The ELISA kits were procured from Eurofins Abraxis, INC (Part Number 520011OH). Water samples were divided into two subsamples. The first sample was filtered through a GFC filter (Whatman, UK) and immediately analyzed by the ELISA method; it represented the extracellular microcystin levels. The second sample was exposed to three freeze/thaw cycles before filtration to lyse and to release the toxin from the cells, and thus the measured microcystin levels represented the total microcystin concentrations (i.e the intra and extra cellular). Microcytin concentrations were determined by fitting a semi-log curve of %Bo for each standard; where $\%Bo = \frac{B}{Bo} \times 100$, versus the corresponding microcystin concentrations. B is the mean absorbance value for each standard while B_0 is the mean absorbance value for the zero standard. A six standards ELISA kit ranging between 0.0 ppb and 5 ppb was used to calibrate the semi-log curve according to the procedure developed by Eurofins Abraxis, INC (Abraxis). Preprogramed Excel macros developed by Eurofins Abraxis, INC generated and validated the results. Note that the samples that resulted in concentrations higher than standard 5 (5.0 ppb) were flagged and subsequently diluted with the sample diluent (provided with the Eurofins Abraxis Kit) before they were reanalyzed. Dilution ranged between 1 and 20 times.

2.2.2 Chlorination

Algaecide treated samples were collected every 24 hrs from the inhibition flasks over 4 days and subjected to chlorination. The collected samples were first filtered using Whatman 5.5 cm grade 4 cellulose filter paper before being exposed to two different chlorine dosage. The filter provides excellent retention of coarse particles (20-25µm), which is comparable to sand filtration used

with conventional water treatment processes prior to chlorination. The two chlorine dosages were generated from a stock solution of 5.6% sodium hypochlorite (NaOCl, Ficher Scientific). For each of the chlorination experiments, the residual chlorine levels were determined after 30 mins contact time using a portable spectrophotometer (Spectrophotometry, HACH DR/900). After 30 mins of contact time, samples were quenched with sodium thiosulfate (Na_2SO_3) before the chlorophyll-a, MC-LR, cell integrity (flow cytometry), TOC/DOC, and THM levels were assessed. TOC, DOC, and THM levels were measured since chlorine can react with certain types of naturally occurring organic materials (NOMs) in water to form disinfection by-products (DBPs), some of which are toxic compounds, such as THMs. TOC is the main surrogate parameter for the measurement of NOM found in the water sample, whereas DOC is measured after a filtration step with 0.45 µm filter diameter. Both DOC and TOC levels were measured using a TOC analyzer (Shimadzu, Columbia, MD USA) according to Standard Methods 531B (Eaton & Franson, 2005). Suspended Solids (SS) is the difference between TOC and DOC and was calculated as such. THM levels were measured using gas chromatography with an electron detector (GC/ECD) using EPA Method 551.1 (USEPA, 1995).

CHAPTER 3

RESULTS

3.1 Algaecide treatment

3.1.1 Chlorophyll-a

H_2O_2 proved to be effective in reducing chlorophyll-a concentrations (Figure 5). Even with the lowest treatment concentration (1 mg/L of H_2O_2), Chl-a levels were found to have dropped on average by 39% per day, assuming a first order decay process (Figure 5.b). With the 7 mg/L of H_2O_2 application rate, the drop in Chl-a levels was slightly higher, reaching 41% per day (Figure 5.c). At the highest tested dose of 10 mg/L of H_2O_2, the Chl-a concentrations dropped at a rate of 42% per day (Figure 5.d). Ultimately, the concentrations achieved by day 4 were found not to have any statically significant difference with regards to their values across the three tested dosages (Figure 5). As for the Chl-a drop achieved by applying 1 mg/L of $CuSO_4$, the mean daily reduction rate was 42% (Figure 5.e). Note that even in the control samples, Chl-a levels dropped on average by 13% per day. Table 1 summarizes the estimated daily reductions in Chl-a levels across the 4 tested algaecide dosages. Interestingly, the ability to represent the Chl-a decrease over time as a first order decay process degraded with higher dosages of algaecide (Table 1).

Table 1 Percent drop of chlorophyll-a levels per day post algaecide application assuming first order decay

	Percent Drop (%)	R-squared (%)
Control	13	96
H_2O_2 (1mg/L)	39	97
H_2O_2 (7mg/L)	41	85
H_2O_2 (10mg/L)	42	75
$CuSO_4$ (1mg/L)	42	82

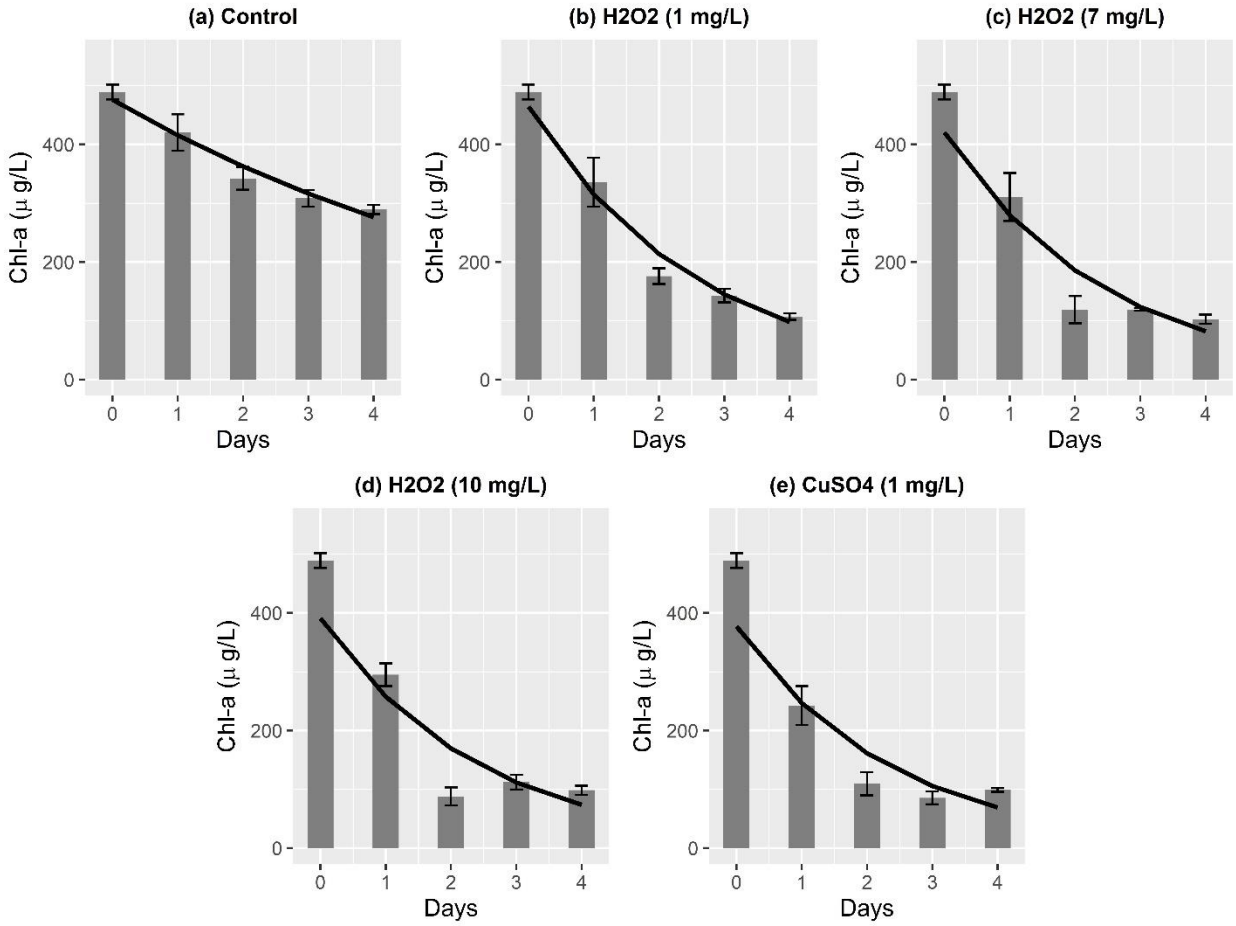


Figure 5 Chlorophyll-a levels post algaecide treatment. The error bars represent ± 1 standard deviation calculated from the triplicate samples. (a) Control; (b) 1mg/L of H2O2; (c) 7 mg/L of H2O2; (d) 10 mg/L of H2O2; (e) 1 mg/L of CuSO4. The error bars represent the standard deviation.

3.1.2 Flow Cytometry

The mean percentage of dead cells (SYTOX+) after 48 hrs of algaecide application ranged between 56% for 1 mg/L of H_2O_2 and 92% for $CuSO_4$ 1 mg/L (Figure 6). In comparison the percentage of dead cells in the control was around 49%. As for the percentage of alive colonial cells measured after 48 hrs of treatment, it dropped with increased H_2O_2 dosages (Figure 6). Their percentage was 9.4% at 1 mg/L of H_2O_2, 5.4% at 7 mg/L of H_2O_2, and reached a minimum of 3% at 10 mg/L of H_2O_2 (Figure 6). The percentage of colonial cells following the 1 mg/L $CuSO_4$ treatment was found to be 5%. Note that the colonial percentage in the control sample was 14% after 48 hrs. The increased drop in the percentage of colonial cells with increasing algaecide dosages is due to the breakdown of colonies into dead and unicellular cells. With regards to the alive unicellular *Microcystin* cells, their percentage after 48-hrs of algaecide treatment was found to be 34%, 12%, 10%, and 3% for the 1, 7 and 10 mg/L of H_2O_2 as well as at 1 mg/L $CuSO_4$ application rate respectively (Figure 6). Their percentage under the control was around 37%. Interestingly, the relative percentage of colonial alive cells to that of the unicellular alive cells increased with higher algaecide dosage. This indicates that a higher percentage of unicellular cells tend to die as compared to those in colonies. This is expected as colonial cells are protected by a boundary layer that contains high levels of extracellular polymeric substances (EPS), which in return can buffer the effects of algaecides (Kehr et al., 2006; Ma et al., 2014; Pereira et al., 2009; Wu, Gan, Huang, & Song, 2007; Yang, Kong, Shi, & Cao, 2006). Following 96 hrs of algaecide application, the percentage of dead cells (SYTOX +) increased under all treatments (Figure 7). It ranged between 73% under the 1 mg/L of H_2O_2 and 91% for the $CuSO_4$ 1 mg/L treatment. The percentage of dead cells in the control was 54% over the same time period (Figure 7). The percentage of colonial cells decreased with higher dosages of H_2O_2;

the percentage at the 1 mg/L dose was 9%, 8% at the 7 mg/L, and 4% at the 10 mg/L dose (Figure 7). At the 1 mg/L of $CuSO_4$, the percentage reached 4%. In comparison the percentage of colonial cells in the control was around 12%. With regards to the percentage of unicellular cells, they showed a similar pattern to that of the colonial. Their percentages were found to be 18%, 17%, 9%, and 5% for the 1, 7, and 10 mg/L of H_2O_2 and the 1 mg/L of $CuSO_4$, respectively. Note that the unicellular percentage in the control sample was 33% after 96 hrs (Figure 7). Similar to what was observed at 48 hrs, the percentage of colonial cells to that of unicellular cells increased with algaecide dosage, indicating a disproportionately higher drop in the latter.

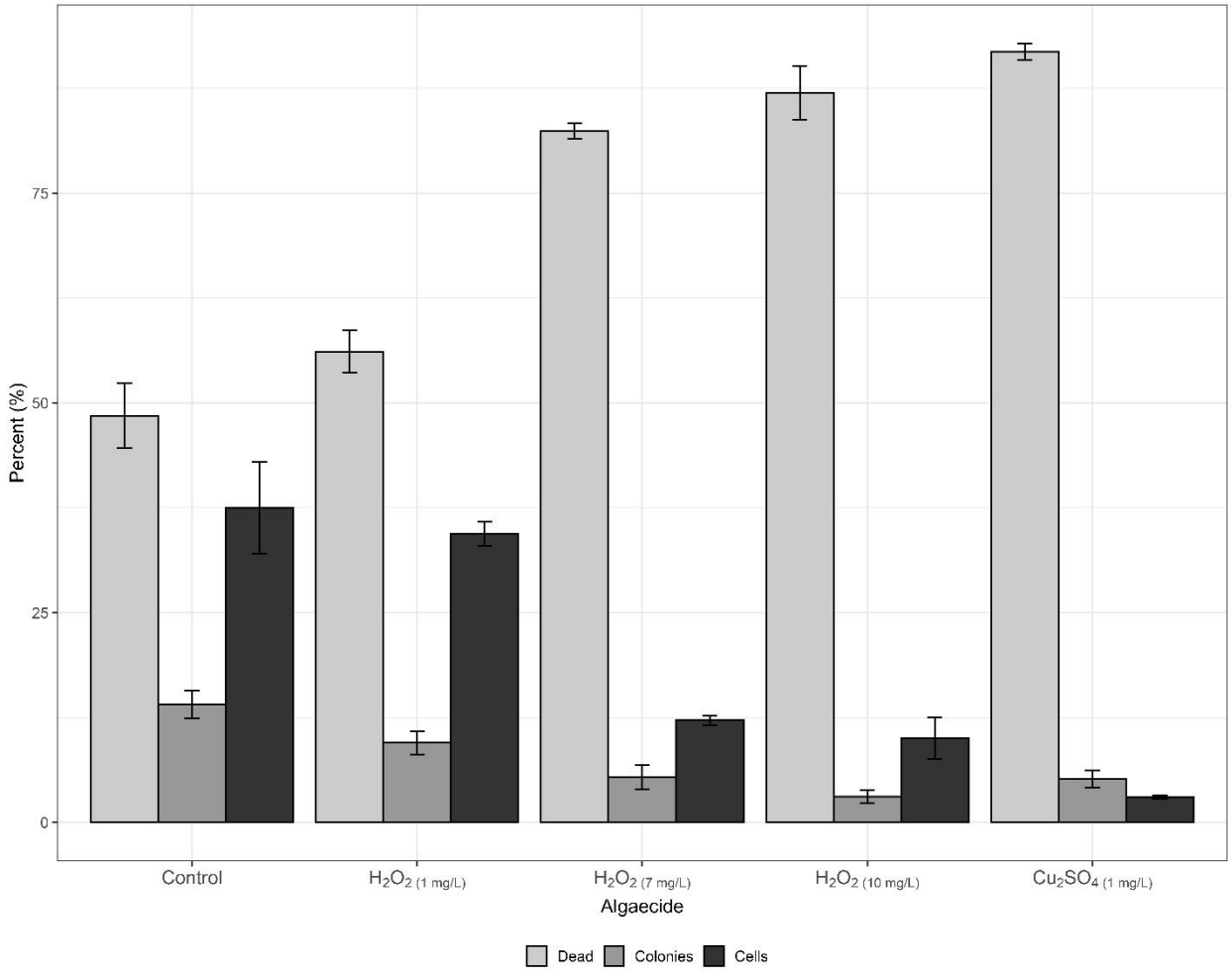


Figure 6 Flow cytometry results showing percentage of dead, colonial, and unicellular Microcystis cells at 48 hrs in control samples and under different algaecide concentrations.

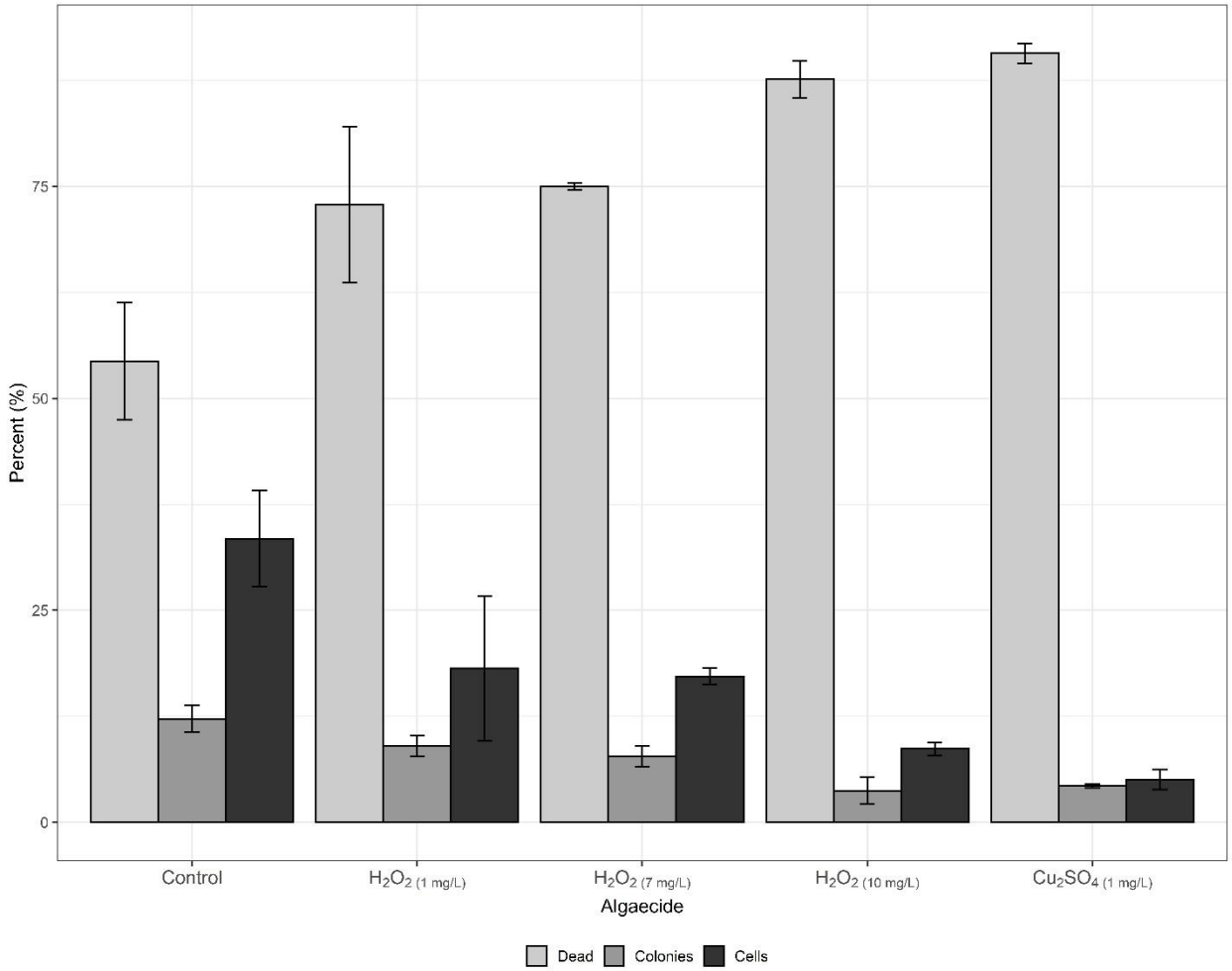


Figure 7 Flow cytometry results showing percentage of dead, colonial, and unicellular Microcystis cells at 96 hrs in control samples and under different algaecide concentrations

3.1.3 MC-LR

To assess the risk of toxin release from *Microcystis aeruginosa* as a result of algaecide application, the total, extracellular, and intracellular MC-LR concentrations were measured across all algaecide dosages. Before algaecide treatment the intracellular MC-LR concentration in the feed water was found to be 61.9 µg/L, while the initial concentration of the extracellular MC-LR was 13.8 µg/L. Over time, the intracellular MC-LR levels in the control samples were found to decrease at a rate of 29% per day (Figure 8.a). This drop can be explained by the natural cell degradation process (discussed in the previous section, whereby Chl-a concentrations

(Figure 5) and the percentage of alive cells (Section 3.1.2) were found to have decreased in the control samples over time). By day 4, the average intracellular concentrations in the control samples was 19 µg/L. While it was expected that the release of the intracellular toxin would cause an increase in the extracellular MC-LR levels, the results showed that the extracellular levels also dropped over time. Overall, in the control samples the average total MC-LR level decreased from 75.8 µg/L at the start of the experiment down to 21.7 µg/L by day 4.

When comparing the toxin levels in the control samples to those measured post H_2O_2 and $CuSO_4$ treatment, as expected the decrease in intracellular toxin content was higher post-treatment. For the 1 mg/L $CuSO_4$ concentration, almost all cells were found to have lost their integrity after 48 hrs of exposure (Figure 8.e). The impact of cell rupture on the toxin release was evident by the large decline in the measured intracellular MC-LR levels, which was estimated to be around 4.5%/hr. At the end of day 4 the average level of intracellular MC-LR was found to be 0.889 µg/L. As for the H_2O_2 application, the measured levels of the intracellular toxins were found to have dropped by 39, 42, and 61%/day for the three applied dosages of 1, 7 and 10 mg/L respectively (Figure 8.b,c,d). With these high levels of daily decay rates, the largest drop in concentration occurs on day 1 and mainly stabilizes afterwards.

With regards to the extracellular levels, a similar pattern of fast degradation of the toxin was observed in the first 24 hrs for all H_2O_2 treatments as well as for the 1 mg/L of $CuSO_4$. Between 65 and 75% of the extracellular concentrations measured prior to treatment were found to have been degraded within the first 24 hrs. Afterwards, the MC-LR concentrations remained relatively stable across the remaining 3 days. The extracellular MC-LR levels after 96 hrs across the four treatments ranged between 2.1 and 2.5 µg/L (Figure 8). It should be noted that in the absence of a natural biological pathway to breakdown the toxins in the water, we would have expected that

the extracellular MC-LR levels to have increased in magnitude over time as a result of cell lysis. While H_2O_2 is a strong oxidant and could have played a role in oxidizing the extracellular toxins, the fact that similar decay rates were observed in the control indicates that the relative role that the oxidant plays is probably minor as compared to natural degradation by bacteria.

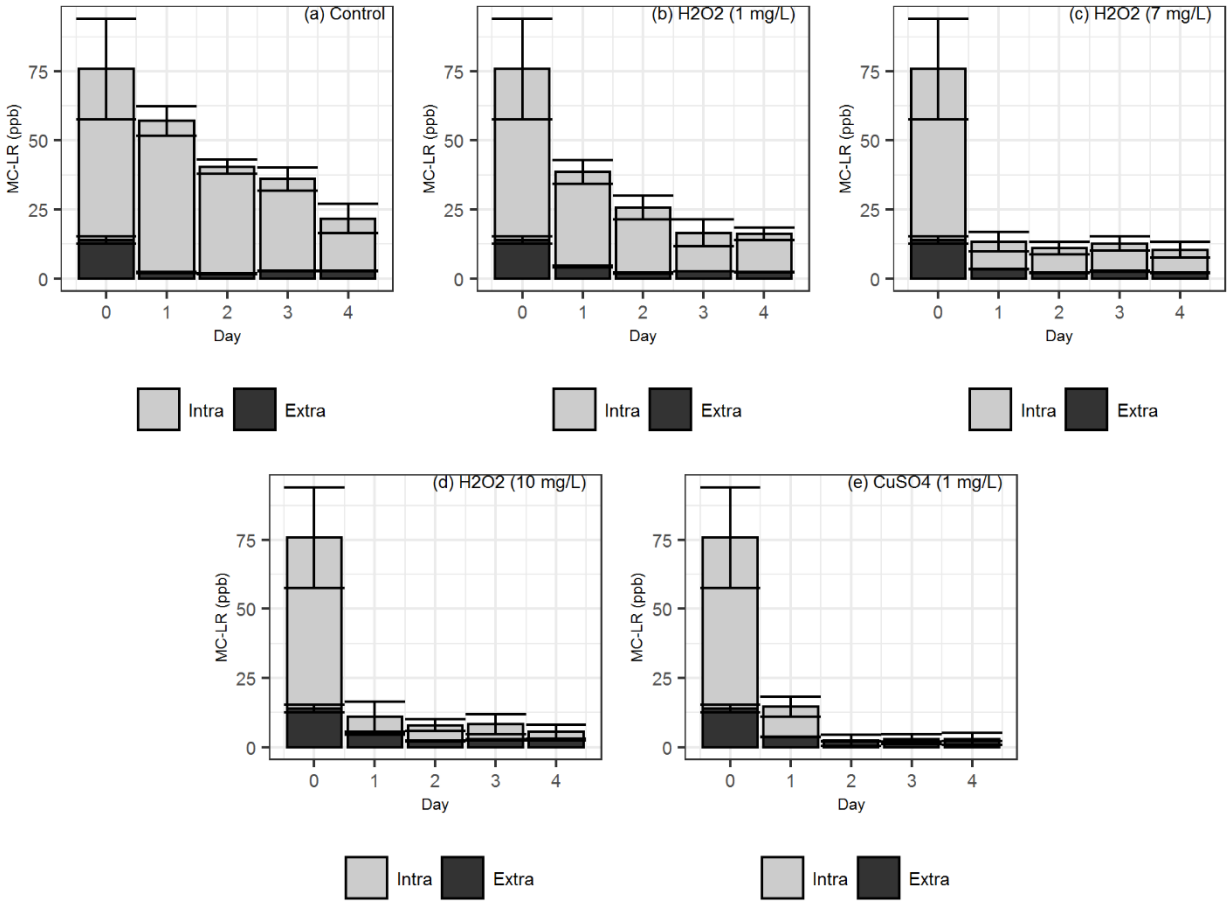


Figure 8 Intracellular and extracellular MCs concentrations after treatment (up to 4 days contact time) under different algaecide concentrations. (a) Control; (b) 1mg/L of H2O2; (c) 7 mg/L of H2O2; (d) 10 mg/L of H2O2; (e) 1 mg/L of CuSO4. The error bars represent the standard deviation.

3.2 Chlorinating algaecide-treated water

The algaecide-treated water samples were subjected to a filtration step that was followed immediately by a 30 mins chlorination period. The effectiveness of the filtration and chlorination process was assessed over time by varying the instance that they were applied post algaecide

application. We explored differences in their effectiveness when they were applied at 24, 48, 72, or 96 hrs from the time the algaecides were applied. This allowed us to assess the optimal time that filtration and chlorination can be applied as a function of the algaecide type and dose used as well as the utilized chlorine dose. The control samples also underwent the same process of filtration and chlorination at the above defined times. The filtration step is meant to mimic typical water treatment processes that opt for filtration and/or sedimentation of raw lake water. Filtration is expected to reduce the suspended organic content resulting from algaecide use and to trap some of the *Microcystis* cells and colonies. Chlorination was restricted to 30 minutes only.

3.2.1 Chlorophyll-a

The percent reduction in Chl-a levels attributed to the filtration process was tracked over time by comparing the concentrations achieved post algaecide treatment with those measured after the samples underwent filtration. Filtering the samples after 24 hrs from algaecide application resulted in Chl-a levels reductions that varied between 57.5%, 61%, and 73% for H_2O_2 1, 7 and 10 mg/L respectively, and 71.9% for $CuSO_4$ for the first day. Percent reductions in Chl-a levels when filtration occurred after 96 hrs of algaecide application were found to have increased to 78.4% with the 1 mg/L H_2O_2 dose, 81.4% with the 7 mg/L H_2O_2 dose, and 83.5% with the 10 mg/L H_2O_2 dose, and 92.5% with 1 mg/L $CuSO_4$. The increase in the percent drop of Chl-a levels with time is probably due to the increased percentage of colonial cells to unicellular cells over time. Filtration is expected to selectively inhibit the passage of large colonies through the filter. Table 2 summarizes the reductions in Chl-a levels that are attributed to filtration over a period of 4 days.

Immediately after the algaecide-treated sampled were filtration, they underwent chlorination. Hence, chlorination occurred either after 24, 48, 72, or 96 hrs from the time the algaecides were

applied. Two different dosages of chlorine, namely 5 and 10 mg/L were used. Each sample was limited to a 30 min contact time. Table 2 compares the chlorination induced reductions in Chl-a levels to those attributed to algaecide application and filtration. As can be seen, the percent drop in Chl-a levels that can be directly attributed to the chlorination process appears to be modest, when compared to the reductions attributed to the algaecides as well as to the filtration process. Nevertheless, the impact of chlorine on the reduction of Chl-a levels was most significant for the samples that were chlorinated after 24 hrs of algaecide treatment. This is to be expected, as Chl-a levels were still elevated after 24 hours of algaecide treatment and as such Cl_2 had the opportunity to react with non-lysed *Microcystis* cells. Moreover, the reductions achieved by the 10 mg/L Cl_2 dose were consistently higher than those achieved by the 5 mg/L dose across all days.

Table 2 Chlorophyll-a concentrations and their percent reductions following algaecide treatment, filtration, and chlorination

	Time			
	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**
	Chl-a concentration in µg/L in control			
	420.3	342.1	308.3	289.2
	Time since algaecide application			
	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**
	Chl-a concentration in µg/L after algaecide treatment (in % reduction [a])			
H_2O_2 (1mg/L)	335.6 (20.1)	175.9 (48.6)	143.0 (53.6)	107.3 (62.9)
H_2O_2 (7mg/L)	310.5 (26.1)	119.1 (65.2)	119.4 (61.3)	102.7 (64.5)
H_2O_2 (10mg/L)	295.0 (29.8)	88.0 (74.3)	112.2 (63.6)	98.5 (65.9)
$CuSO_4$ (1mg/L)	242.6 (42.3)	109.2 (68.1)	85.5 (72.3)	98.8 (65.9)
	Chl-a concentration in µg/L after filtration (in % reduction [b])			
H_2O_2 (1mg/L)	142.7 (57.5)	71.4 (59.4)	41.5 (71.0)	23.2 (78.4)
H_2O_2 (7mg/L)	121.4 (60.9)	46.6 (60.8)	32.1 (73.1)	19.1 (81.4)
H_2O_2 (10mg/L)	79.8 (73.0)	35.4 (59.7)	28.4 (74.7)	16.2 (83.5)
$CuSO_4$ (1mg/L)	68.2 (71.9)	36.1 (67.0)	16.0 (81.2)	7.3 (92.5)
	Chl-a concentration in µg/L after chlorination with 5 mg/L (in % reduction [c])			
H_2O_2 (1mg/L)	41.1 (71.2)	51.9 (27.2)	28.2 (32.0)	9.8 (57.6)
H_2O_2 (7mg/L)	15.9 (68.6)	32.2 (33.3)	24.5 (29.7)	7.7 (67.6)
H_2O_2 (10mg/L)	38.1 (55.3)	31.1 (27.8)	22.5 (29.4)	6.2 (63.4)
$CuSO_4$ (1mg/L)	22.1 (42.4)	15.5 (43.8)	18.0 (49.7)	5.7 (28.2)
	Chl-a concentration in µg/L after chlorination with 10 mg/L (in % reduction [c])			
H_2O_2 (1mg/L)	35.6 (88.8)	25.6 (54.8)	20.0 (40.8)	5.9 (66.5)
H_2O_2 (7mg/L)	20.1 (81.8)	23.7 (66.7)	11.4 (43.8)	5.7 (70.0)
H_2O_2 (10mg/L)	39.2 (74.7)	20.2 (32.9)	8.0 (59.9)	5.2 (64.5)
$CuSO_4$ (1mg/L)	32.1 (52.8)	14.3 (60.1)	5.9 (62.9)	3.9 (45.9)

[a]: % reduction from control
[b]: % reduction from algaecide treated samples
[c]: % reduction from algaecide treated and filtered samples

3.2.2 Flow cytometry

After 48 hrs of algaecide treatment and following filtration, the percentage of dead cells reached 73%, 84%, 91%, and 95% at 1, 7, 10 mg/L of H_2O_2, and 1 mg/L of $CuSO_4$ respectively. Note that the increase in the percentage of dead cells post filtration is largely due to the selective filtration by size, whereby dead cells and unicellular cells tend to have a higher percentage of passing through the filter. The additional exposure to 30 mins of chlorine treatment increased the percentage of dead cells across all algaecides due to the lysing effect that Cl_2 had on the remaining *Microcystis* cells. When treated with 5 mg/L of Cl_2, their percentage ranged between a minimum of 79 % for the 1 mg/L of H_2O_2 and a maximum of 91 % under the 1 mg/L of $CuSO_4$. The percentage of dead cells increased further when the Cl_2 treatment was 10 mg/L; the percentage ranged between a minimum of 91% for 1 mg/L of $CuSO_4$ and 97% for the 10 mg/L of H_2O_2 application. With regards to the percentage of colonial cells, their percentage post-filtration and after 48 hrs of algaecide treatment dropped significantly. It ranged between a minimum of 1% (for the 10 mg/L of H_2O_2) and a maximum of 4% (for the 1mg/L of H_2O_2 and 1 mg/L of $CuSO_4$). Post-chlorination, the percentage of colonial cells remained largely unchanged as compared to those reported post-filtration. With respect to the unicellular alive cells, their overall percentage post-filtration decreased and ranged between a minimum of 2% for the $CuSO_4$ application samples and 23% for the 1 mg/L of H_2O_2 treatment. After 30 mins contact time with Cl_2, the percentage of unicellular cells decreased with increased Cl_2 dosage across all algaecides. Their percentages ranged between 2 and 15% with 5 mg/L of Cl_2 and between 1 and 5% with 10 mg/L of Cl_2.

After 96 hrs of algaecide application, the percentage of dead cells in the filtered samples was 66%, 83%, 90%, and 92% for the 1, 7, and 10 mg/L of H_2O_2 as well as 1 mg/L of $CuSO_4$ respectively. The additional exposure of the samples at 96 hrs to Cl_2 over 30 min resulted in a

further increase in the percentage of dead cells. Their percentage ranged from around 85% for the 1 mg/L H_2O_2 treated samples to more than 98% for the $CuSO_4$ treated samples. Note that increasing the chlorine dosage from 5 to 10 mg/L did not seem to have a major advantage on the percentage of dead cells in a given treated sample. As for colonial cell, their percentage decreased significantly after filtration across all algaecides; their percentage ranged between 1% and 3%. Their percentage remained largely similar when the filtered samples underwent further chlorination for 30 mins irrespective of dose. As for the percentage of unicellular cells, their percentage after filtration ranged between 5% for $CuSO_4$ and 31% under the lowest dose of H_2O_2. Moreover, a further reduction was seen when the samples were chlorinated for 30 mins across the 4 algaecide dosages. The percentages of healthy unicellular cells after chlorination at 96 hrs post-algaecide treatment ranged between 0.4% and 12%, with no comparable difference between the two chlorine dosages.

3.2.3 MC-LR

Total MC-LR levels decreased post-filtration across all algaecide dosages. The percent drop ranged between 10% for the samples that were exposed to 1 mg/L of $CuSO_4$ for 48 hrs and 70% for the samples that were exposed to 7 mg/L of H_2O_2 for 96 hrs (Appendix D). This drop is largely attributed to the entrapment of the colonial cells on the filter. As such, the drop in the total MC-LR levels was correlated to the drop in the measured intra-cellular toxin concentrations. The highest levels of total MC-LR after filtration and post-chlorination were for the samples that were treated with 1 mg/L H_2O_2 (Figure 9.a). Most of those samples had concentrations of Total MC-LR in excess of 5 ppb. Moreover, a big part of the toxin was still intracellular across the 4 days of treatment. At the higher dosage of 7 mg/L and 10 mg/L of H_2O_2, the measured MC-LR levels were found to be significantly lower (Figure 9.b,c). At the 7

mg/L, the Total MC-LR level exceeded the 5 ppb level only when the samples were filtered but not chlorinated. Moreover, a large portion of the toxin was still intracellular. Post chlorination, all total MC-LR concentrations had levels well below 5 ppb. At the highest H_2O_2 dose (10 mg/L), all concentrations were below the 5 ppb level even when they were only filtered (Figure 9,c). Chlorinating these samples resulted in a small decrease in the toxin level. As expected, the percentage of the extracellular MC-LR was found to increase as the dose of H_2O_2 increased from 1 to 10 mg/L. This is largely explained by enhanced cell lysing at higher dosages. The MC-LR levels for the filtered and chlorinated samples that were treated with $CuSO_4$ tended to have the lowest toxin level of all samples, with the exception of the first 24 hrs (Figure 9.d). Additionally, for the $CuSO_4$ treated samples, most of the measured toxin was extracellular, which further reinforces the lysing potential of $CuSO_4$. It should be noted that the fact that the $CuSO_4$ samples had the lowest MC-LR levels is an interesting finding, as one would expect that samples exposed to high levels of H_2O_2 would benefit from the oxidative potential of H_2O_2 to degrade the MC-LR further. This finding may indicate that the oxidative removal of MC-LR by H_2O_2 may have be minor as compared to the deactivation of the toxin by naturally occurring bacteria in the water. With regards to the effect of Cl_2 on the toxin level, the results show that chlorination appears to play a positive role in reducing toxin levels in the finished water. Total toxin levels post chlorination were generally significantly lower as compared to those measured immediately after filtration. Yet looking closely at the resulting extracellular MC-LR levels, one can see that at the 7 and 10 mg/L H_2O_2 the levels of extracellular MC-LR levels increased post chlorination as compared to the levels pre-chlorination (Figure 9.b,c). This is probably due to increased cell-lysising. This pattern was observed also partially in the 1 mg/L H_2O_2 treated samples, particularly at the higher chlorine dosages. With the exception of the 1 mg/L H_2O_2 samples, there

was no marked difference in the toxin levels between the 5 and 10 mg/l Cl_2 applications (Figure 9.a).

Overall, it appears that opting to delay the filtration and chlorination process of algaecide-treated waters is advantageous when it comes to ensuring that the finished water has a lower level of MC-LR. This pattern was apparent across the 7 and 10 mg/L H_2O_2 dosages and the 1 mg/L $CuSO_4$ level. That pattern was less apparent for the 1 mg/L H_2O_2 dose. Nevertheless, the results show that across all the tested algaecide dosages and irrespective of when the filtration and chlorination process was implemented, none of the samples were able to achieve the 1 μg/L WHO (2003) Guideline value for Microcystin-LR. This highlights the high MC-LR related risks that consumers may be exposed to when water establishments rely on surface water bodies that are prone to HABs.

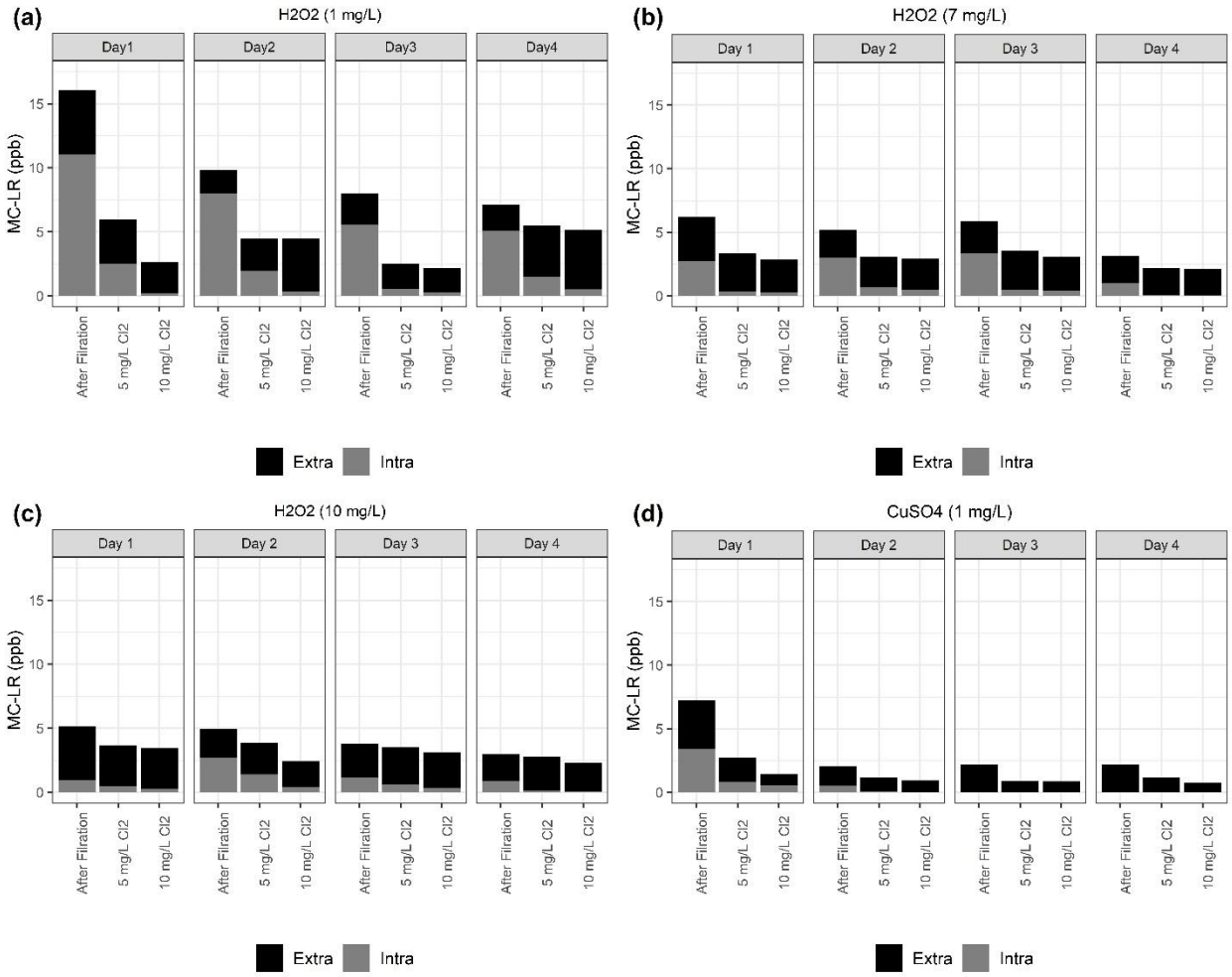


Figure 9 Intracellular and extracellular MCs concentrations after filtration treatment and after filtration and chlorination treatment (up to 4 days contact time) under different algaecide concentrations. (a) 1mg/L of H2O2; (b) 7 mg/L of H2O2; (c) 10 mg/L of H2O2; (d) 1 mg/L of CuSO4.

3.2.4 Residual Chlorine

Throughout the chlorination experiments, the residual chlorine levels didn't exceed the 2 mg/L across all algaecide and chlorine dosages applied and irrespective of the time at which the chlorine was added. Yet, we observed that overall the residual chlorine levels tended to increase with higher chlorination levels and higher H_2O_2 dosages. The application of chlorine on samples exposed to the lowest concentration of H_2O_2 (1 mg/L) recorded very low chlorine residual irrespective of the day that chlorine was added. For all the H_2O_2 treated samples, the highest recorded residual Cl_2 concentrations occurred when chlorination occurred after 24 hrs post

algaecide application (Table 3). Since $CuSO_4$ is a weaker oxidant as compared to H_2O_2, chlorine was highly consumed after 30-mins of contact time across all days.

As expected, the pH of the chlorinated waters had a role in determining the amount of residual chlorine levels in the treated waters. In our samples (refer to Appendix B), the pH values tended to increase with the dosages of H_2O_2 as a result of the formation of the hydroxyl radical (OH) (Huo, Chang, Tseng, Burch, & Lin, 2015). Moreover, pH values increased post-chlorination. Highest pH levels were recorded when the 10 mg/L Cl_2 dose was used. Given that the effectives of chlorine is highly related to the pH, the highest residual levels occurred on day 1 and under the highest dose of H_2O_2 used (Table 3).

Table 3 Residual chlorine concentrations after 30-mins contact time for different application times

	Time between algaecide application and chlorination			
	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**
	Residual Chlorine concentration in mg/L after chlorination with 5 mg/L			
H_2O_2 (1mg/L)	0.21	BDL*	0.1	0.02
H_2O_2 (7mg/L)	0.57	0.26	0.02	0.06
H_2O_2 (10mg/L)	0.78	0.14	0.03	0.24
$CuSO_4$ (1mg/L)	0.32	0.16	0.01	0.04
	Residual Chlorine concentration in mg/L after chlorination with 10 mg/L			
H_2O_2 (1mg/L)	0.37	0.47	0.13	0.04
H_2O_2 (7mg/L)	1.4	1.1	0.04	0.21
H_2O_2 (10mg/L)	1.8	0.34	0.07	0.49
$CuSO_4$ (1mg/L)	0.9	0.11	0.04	0.06

*BDL: below detection limit <0.01 mg/L

3.2.5 TOC/DOC and THM

As can be seen from Figure 10, both the measured DOC and the calculated SS concentrations tended to drop following filtration and the addition of chlorine as compared to the levels measured prior to filtration (Figure 10). With regards to the formation of THMs post

chlorination, their levels were found to exceed the WHO guideline value of 1µg/L when chlorination occurred within 24 hrs of algaecide application across all tested algaecide dose combinations. THM concentrations were well below the WHO standard when chlorination occurred more than 24 hrs from the time that H_2O_2 was applied, irrespective of the chlorine and H_2O_2 dose. For $CuSO_4$, THM levels in excess of the WHO standard were observed when chlorination occurred either 24 hrs or 48 hrs from the time that the algaecide was added (Table 4). Levels on day 3 and 4 post algaecide application were well below the WHO standard.

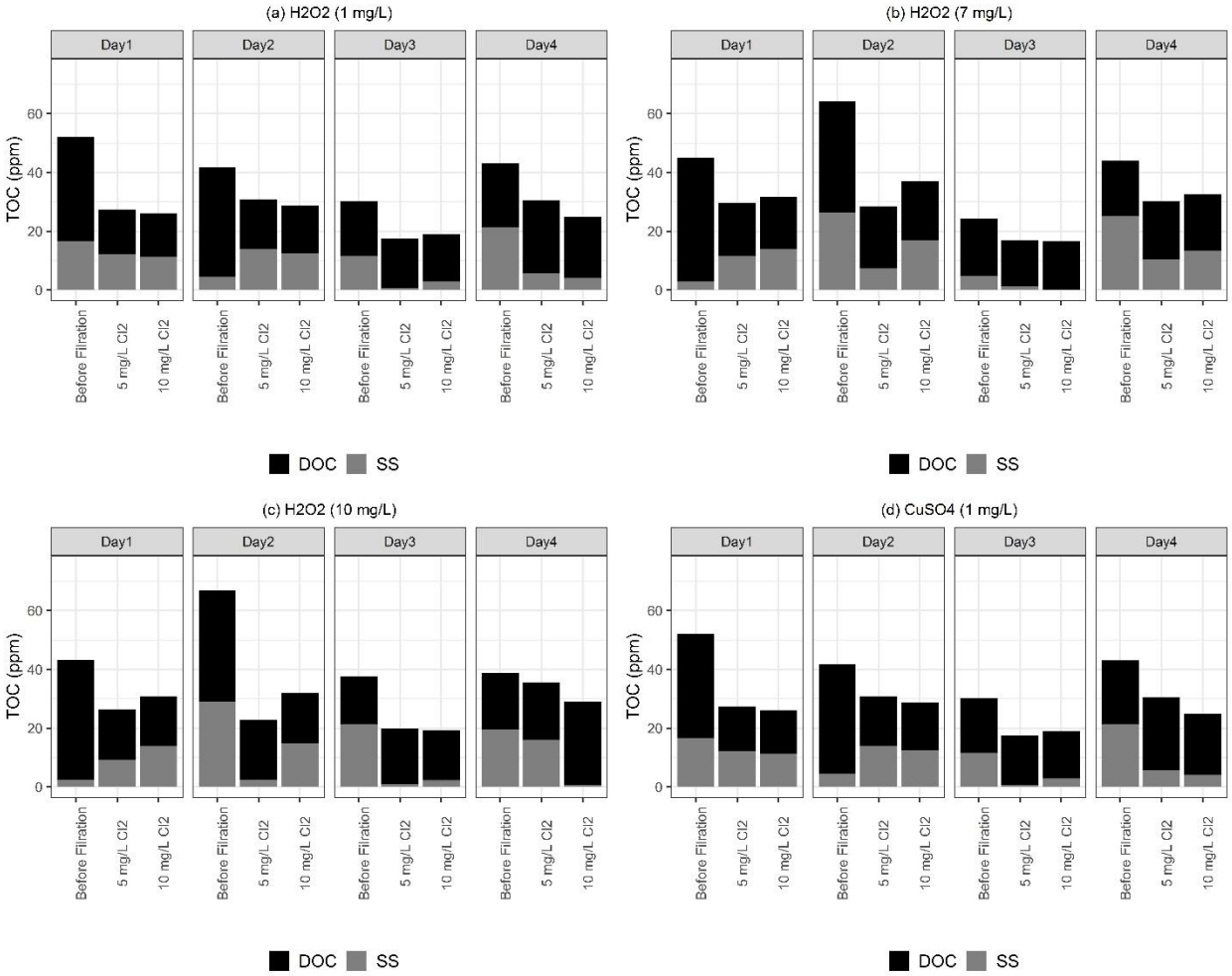


Figure 10 DOC and Suspended solids before filtration and after chlorination treatment (up to 4 days contact time) under different algaecide concentrations. (a) 1mg/L of H_2O_2; (b) 7 mg/L of H_2O_2; (c) 10 mg/L of H_2O_2; (d) 1 mg/L of $CuSO_4$.

Table 4 THMs levels measured in treated samples post algaecide application and after chlorinating for 30 mins

	Time between algaecide application and chlorination			
	24 hrs	**48 hrs**	**72 hrs**	**96 hrs**
	THM levels in µg/L after chlorination with 5 mg/L			
H_2O_2 (1mg/L)	1.35	0.025	0.025	0.28
H_2O_2 (7mg/L)	1.42	0.14	0.06	0.27
H_2O_2 (10mg/L)	0.59	0.17	0.08	0.11
$CuSO_4$ (1mg/L)	1.09	2.28	0.11	0.09
	THM levels in µg/L after chlorination with 10 mg/L			
H_2O_2 (1mg/L)	9.9	0.38	0.16	0.26
H_2O_2 (7mg/L)	4.67	0.8	0.025	0.14
H_2O_2 (10mg/L)	1.84	0.52	0.06	0.12
$CuSO_4$ (1mg/L)	4.41	1.75	0.025	0.025

CHAPTER 4

DISCUSSION

4.1 $CuSO_4$

The application of 1 mg/L of $CuSO_4$ was able to achieve a maximum reduction of 83% in terms of Chl-a levels after 72 hrs of treatment. Previous studies have reported higher removal rates with the application of 1 mg/L of $CuSO_4$. Dia et al. (2019) and Fan et al.(2013) both reported reductions in excess of 95% after 4 days of exposure. However, both of these studies started with lower Chl-a concentration (3 times less) and lower cell densities (10 times less). The Chl-a results in this study were in full agreement with the flow-cytometry readings, whereby we observed that the percentage of dead *Microcystis* cells in the treated samples reached ~90% after 48 hrs from the $CuSO_4$ application. These results are consistent with the results reported by Fan, Ho, et al. (2013), who reported that the percentage of lysed cells reached 98% after 48 hrs of exposure to 1 mg/L of $CuSO_4$. Interestingly, the flow cytometry results also showed that $CuSO_4$ had a strong impact on cell complexity, resulting in the breakdown of colonial cells into unicellular cells.

The effectiveness of copper sulfate often comes at the expense of increased risks of intracellular toxin release from the lysed cells. In this study, we found that 96% of *Microcystis* cells had lysed after 48 hrs of treatment, which is consistent with the results reported by Fan et al. (2014), who observed complete cell lysing after 48 hrs of exposure. Looking at the intracellular MC-LR levels, we can see the gravity of cell rupture on toxin release. The intracellular toxin was found to have dropped on average by around 4.5% per hour over the 4 days of the experiment. This

resulted in reducing the intracellular MC-LR levels from 61.9 µg/L before $CuSO_4$ application to 0.9 µg/L by day 4. The drop in the intracellular MC-LR levels was expected to be accompanied by a large increase in the extracellular MC-LR levels (Fan et al., 2014; Jones & Orr, 1994; Kenefick, Hrudey, Peterson, & Prepas, 1993). However, in this study this was not the case. We observed that the extracellular toxin levels degraded quickly, in a similar fashion to the control samples. The degradation of extracellular MC-LR in this study is most probably attributed to naturally occurring indigenous microbial flora in the raw lake water that have been shown to be effective in breaking down MC-LR. Of particular importance is the gram-negative phylum of *Proteobacteria* that are known to be common in freshwater systems (Best et al., 2002; Dziga, Wasylewski, Wladyka, Nybom, & Meriluoto, 2013). Nevertheless, even with the significant degradation of MC-LR in the treated waters, the total MC-LR levels post $CuSO_4$ treatment remained consistently higher than 2 µg/L across the 4 days of exposure. As such, using $CuSO_4$ as an algaecide during *Microcystis* blooms can pause risks to the public in the first 4 days of treatment, especially that treated systems would appear to have low Chl-a levels during that time. Prolonging the exposure time to $CuSO_4$ and testing for the MC-LR levels post-treatment in HAB affected systems is thus recommended to ensure that levels fall below the WHO standards (1 µg/L) prior to removing beach closures and/or allowing for direct water use.

The effect of filtering and chlorinating the $CuSO_4$ treated waters was evaluated with regards to the risks associated with exposure to high levels MC-LR as well as the potential for generating THMs in the finished water. Filtration proved to be very efficient in entrapping *Microcystis* cells that remained post algaecide exposure and thus was able to reduce Chl-a concentration by 70 to 90 % (Table 2). These reductions are comparable to the 85% reduction achieved by sand filtration

(Hoeger, Shaw, Hitzfeld, & Dietrich, 2004). Filtration was also efficient in reducing the total MC-LR levels of the copper treated samples by up to 50%; yet the toxin levels post-filtration were still above the recommended WHO standards of 1 µg/L. Jurczak et al. (2005) reported that filtration was able to remove more than 75 % of the MC-LR in the water.

Chlorinating the post-filtrated $CuSO_4$ samples resulted in further reductions to the Chl-a content, with levels dropping by 50% to 60% below the levels achieved post-filtration. Other researchers have reported significant losses in *Microcystis* cell integrity with similar or lower dosages of chlorine. Fan, Ho, et al. (2013) reported achieving 95% cell lysis when *Microcystis* was exposed to Cl_2 doses ranging between 3 and 5 mg/L for 5 mins, while Lin et al. (2009) found that 52% of the cells ruptured after 30 mins of exposure to 6 mg/L Cl_2. Overall, the drop in Chl-a levels following $CuSO_4$ treatment, filtration, and chlorination was between 84 and 98% as compared to their respective controls over the 4 days, with higher reductions observed at 72 hr and 96 hr as well as with higher chlorine doses (Table 2).

Chlorinating the $CuSO_4$ treated samples generated high levels of THMs during the first two days of algaecide application. Levels of THM dropped to 0.025 ppb, when chlorination occurred on day 3 and day 4-post algaecide application. Chlorine addition resulted in the further degradation of MC-LR, both through lysing intracellular toxins and then oxidizing the resulting extracellular MC-LR. Yet, it was found that the recommended level of 1 µg/L MC-LR could not be achieved when chlorination was undertaken during the first 48 hrs. Daly et al. (2007) reported that the total MC-LR levels were reduced by up to 89% after 30 min of exposing a *Microcystis* sample of 10^6 cells/ml to 10.6 mg/L of Cl_2. In this work, the addition of 10 mg/L of Cl_2 to the post-filtered

waters was able to reduce the total MC-LR levels by 80% when the chlorination happened during the first 24 hrs. The percent drop in total MC-LR levels were lower when chlorination happened later; yet this is probably due to the lower pre-chlorine total MC-LR levels. Based on these results, it appears that while the use of chlorine as a post treatment technique is most effective in reducing total MC-LR levels in the first 24 hrs post $CuSO_4$ application, it is recommended to avoid the chlorination of algaecide treated waters with high cell densities due to its tendency to form THM and release intracellular MC-LR. Moreover, it was observed that higher chlorine dosages appear to be needed to ensure a sufficient chlorine residual in the finished water.

4.2 Hydrogen Peroxide

H_2O_2 proved to be largely as effective as $CuSO_4$ in controlling *Microcystis* cells, especially at the higher dosages of 7 and 10 mg/L. At the 10 mg/L dose, 82% removal was achieved by day 2, which was close to the removal reported by Fan et al., (2013; 2014) with a H_2O_2 dos of 10.2 mg/L. Kansole and Lin (2017) reported that 50% to 57% of *Microcystis* cells were inhibited at the 1 and 10 mg/L doses of H_2O_2, respectively. Qian et al. (2012) found that cyanobacterial Chl-a decreased only by 13.9 % after 48 h exposure to the low dose of 1.3 mg/L H_2O_2; yet the experiments were conducted with low *Microcystis aeruginosa* cell density (10^5 cells/ml). Bauzá, Aguilera, Echenique, Andrinolo, and Giannuzzi (2014) reported a reduction of 86% after 48 hrs of treatment with the low dose of 1.67 mg/L of H_2O_2. Reductions with the 1 mg/L dose were around 50% after 48 hrs of H_2O_2 application.

With regards to the MC-LR levels, Fan et al. (2014) was able to reduce the intracellular MC-LR levels by around 40% and the total MC-LR levels by 52% after 24 hrs of exposure to a dose of 10.2 mg/L of H_2O_2. On the other hand, Kansole and Lin (2017) reported that their total MC-LR

was reduced by 38% when the samples were exposed to 10 mg/L of H_2O_2 for 48 hrs with the assistance of UV light. In this study, the reduction in the intracellular MC-LR levels was significantly higher than what was reported by others. It ranged between 40% per day for the 1 mg/L H_2O_2 dose and 60% per day when the dose was increased to 10 mg/L. Similarly, the daily reduction in the total MC-LR levels ranged between 40 and 55% for the 1 and 10 mg/L dosages. Note that the fast reduction recorded for the extracellular MC-LR was also reported by Fan et al. (2014). Ultimately even with these high removal rates, the total MC-LR levels across all the samples that were exposed to hydrogen peroxide were consistently higher than the WHO 1 µg/L provisional standard (Figure 9), especially at low dosages of H_2O_2. As such, the use of H_2O_2 as an algaecide to control cyanobacteria and *Microcystin* should be carefully evaluated when dealing with large blooms, given the risks associated with the toxins. Fan et al. (2014) also expressed similar concerns with regards to H_2O_2 application. Dzinga et al. (2018) reported that while they saw a rapid reduction in *Microcystin* concentrations after H_2O_2 application, concerns persisted with regards to increased MC-LR production. They suggested using H_2O_2 treatment with other oxidizing agents in order to reduce MC-LR levels. Note that while some authors have suggested that higher H_2O_2 dosages may be able to oxidize any extracellular MC-LR; several have warned about the potential impacts on non-target organisms in a natural system (Lürling et al., 2014; Spoof et al., 2020).

Similar to the $CuSO_4$ results, the filtration process reduced the percentage of alive and colonial *Microcystis* cells. Chl-a concentration dropped by 70 to 90% (Table 2) post-filtration. The percent reduction in total MC-LR levels following filtration ranged between 50 to 70%, with most reductions seen in the intracellular MC-LR due to the retention of colonial cells on the filter.

However, the toxin levels post filtration remained high even with the highest dosage of H_2O_2 irrespective of the algaecide exposure time. Chlorinating the post-filtered H_2O_2 samples resulted in a further reduction of Chl-a levels; yet THM levels were high when chlorine was added within 24 hrs of algaecide application. The highest THM level (9.6 µg/L) was recorded when 10 mg/L of Cl_2 were added 24 hrs after the application of 1 mg/l H_2O_2 and (Table 4 & Figure 10). This was probably due to the high percentage of colonial cells still found at the end of day 1 that limited the ability of Cl_2 to react with *Microcystis*. Fan, Rao, Chiu, and Lin (2016) reported that Cl_2 action on colonial cells is slower as compared to unicellular *Microcystis*. This could have provided a higher opportunity for the chlorine to react with the TOC/DOC to form THM. As for MC-LR degradation, chlorine accelerated the degradation of the total MC-LR levels by lysing the cells and releasing the intracellular toxins and then oxidizing the resulting extracellular MC-LR. Yet, the resulting total MC-LR levels, across all of the tested H_2O_2 and Cl_2 dosage combination and across all algaecide exposure time, were still above the recommended WHO standard of 1 µg/L (Appendix D). Therefore, we can conclude that using H_2O_2 alone as an algaecide or combining it with a filtration process and post-chlorination still cannot guarantee the degradation of the toxins in the treated water for safe human or animal consumption.

CHAPTER 5

CONCLUSION AND LIMITATIONS

This study provided the first comprehensive assessment towards assessing the efficacy and risks associated with using different dosages of H_2O_2 and $CuSO_4$ as algaecides to control *Microcystis* blooms, when the treated water is destined for chlorinated afterwards. As such, the percentage of colonial *Microcystis* cells were tracked along with the levels of Chl-a, MC-LR, THM, and residuals chlorine. Additionally, the potential impacts associated with delaying the chlorination process from 24 hrs up to 96 hrs post-algaecide treatment was assessed. The key findings from the study are summarized below:

- Both the 1 mg/L $CuSO_4$ and the 10 mg/L H_2O_2 resulted in the largest inhibition of *Microcystis* cells, disrupting both colonies and unicellular cells. Both also resulted in large reductions in the intracellular and extracellular levels of MC-LR. Yet, total MC-LR levels were still found to be above those recommended by the WHO.
- The 7 mg/L and the 10 mg/L H_2O_2 doses were largely similar with regards to their effectiveness in controlling *Microcystis* and MC-LR levels. The efficacy of 1 mg/L of H_2O_2 was comparatively low.
- Filtration post algaecide application was found to be effective in reducing *Microcystis* cells and colonies. It was also able to reduce the total MC-LR content across all samples, by retaining colonies.
- The combination of $CuSO_4$ with filtration and chlorination proved to be the most effective; it was the only combination able to achieve MC-LR levels below the WHO

standard. Yet, both MC-LR and THM levels were found to be high when chlorination occurred within the first 48 hrs of algaecide application.

- The combination of H_2O_2 with filtration and chlorination resulted in a significant drop in the MC-LR levels but the WHO standard was not met irrespective of when chlorination was implemented. As for risks of generating THM, levels in excess of 1 ppb were observed only when chlorination occurred after the first day of treatment.
- While the water samples were collected from a hypereutrophic reservoir, the conclusions from this study are based on experiments that were conducted under laboratory conditions. As such, care should be taken, and more field-based assessments need to be conducted when a full-scale implementation of the proposed treatment is to be implemented.

Table 5 presents a summary comparison across all algaecide dosages in terms of their efficacy in reducing toxin levels, Microcystis cells and colonies, MC-LR levels, while ensuring that THM levels are below the standard and that the residual chlorine levels are above 0.5 mg/L.

Table 5. Summary of results across algaecides

		Chl-a (µg/L)	MC-LR (µg/L)	Residual Chlorine (mg/L)	THM (µg/L)
		Day 1			
H_2O_2 (1mg/L)	**Cl_2 (5mg/L)**	41.07	5.97	0.21	1.35
	Cl_2 (10mg/L)	15.93	2.61	0.37	9.9
H_2O_2 (7mg/L)	**Cl_2 (5mg/L)**	38.11	3.34	0.57	1.42
	Cl_2 (10mg/L)	22.06	2.84	1.4	4.67
H_2O_2 (10mg/L)	**Cl_2 (5mg/L)**	35.68	3.65	0.78	0.59
	Cl_2 (10mg/L)	20.20	3.45	1.8	1.84
$CuSO_4$ (1mg/L)	**Cl_2 (5mg/L)**	39.30	2.70	0.32	1.09
	Cl_2 (10mg/L)	32.19	1.43	0.9	4.41
		Day 2			
H_2O_2 (1mg/L)	**Cl_2 (5mg/L)**	51.96	4.48	0.05	0.025
	Cl_2 (10mg/L)	32.28	4.50	0.47	0.38
H_2O_2 (7mg/L)	**Cl_2 (5mg/L)**	31.14	3.08	0.26	0.14
	Cl_2 (10mg/L)	15.56	2.90	1.1	0.8
H_2O_2 (10mg/L)	**Cl_2 (5mg/L)**	25.61	3.86	0.14	0.17
	Cl_2 (10mg/L)	23.79	2.40	0.34	0.52
$CuSO_4$ (1mg/L)	**Cl_2 (5mg/L)**	20.29	1.18	0.16	2.28
	Cl_2 (10mg/L)	14.39	0.92	0.11	1.75
		Day 3			
H_2O_2 (1mg/L)	**Cl_2 (5mg/L)**	28.24	2.54	0.1	0.025
	Cl_2 (10mg/L)	24.56	2.18	0.13	0.16
H_2O_2 (7mg/L)	**Cl_2 (5mg/L)**	22.58	3.54	0.02	0.06
	Cl_2 (10mg/L)	18.03	3.10	0.04	0.025
H_2O_2 (10mg/L)	**Cl_2 (5mg/L)**	20.04	3.54	0.03	0.08
	Cl_2 (10mg/L)	11.40	3.11	0.07	0.06
$CuSO_4$ (1mg/L)	**Cl_2 (5mg/L)**	8.07	0.94	0.01	0.11
	Cl_2 (10mg/L)	5.95	0.87	0.04	0.025
		Day 4			
H_2O_2 (1mg/L)	**Cl_2 (5mg/L)**	9.84	5.48	0.02	0.28
	Cl_2 (10mg/L)	7.79	5.14	0.04	0.26
H_2O_2 (7mg/L)	**Cl_2 (5mg/L)**	6.20	2.21	0.06	0.27
	Cl_2 (10mg/L)	5.74	2.10	0.21	0.14
H_2O_2 (10mg/L)	**Cl_2 (5mg/L)**	5.95	2.78	0.24	0.11
	Cl_2 (10mg/L)	5.76	2.29	0.49	0.12
$CuSO_4$ (1mg/L)	**Cl_2 (5mg/L)**	5.29	2.23	0.04	0.09
	Cl_2 (10mg/L)	3.99	1.82	0.06	0.025

Red: values of MC-LR < 1 µg/L, residual chlroine > 0.5 mg/L, THM < 1 µg/L, and Chl-a < 40 µg/L
Green: values of MC-LR > 1 µg/L, residual chlroine < 0.5 mg/L, THM > 1 µg/L, and Chl-a ≥ 40 µg/L

REFERENCES

Abraxis, E. Enzyme-Linked Immunosorbent Assay for the Congener- Independent* Determination of Microcystins and Nodularins in Water Samples In E. Abraxis (Ed.). 124 Railroad Drive Warminster, PA 18974.

American Public Health, A., Eaton, A. D., American Water Works, A., & Water Environment, F. (2005). *Standard methods for the examination of water and wastewater*. Washington, D.C.: APHA-AWWA-WEF.

Atoui, A., Hafez, H., & Slim, K. (2013). Occurrence of toxic cyanobacterial blooms for the first time in L ake K araoun, L ebanon. *Water and Environment Journal, 27*(1), 42-49.

Barrington, D. J., Ghadouani, A., & Ivey, G. N. (2011). Environmental factors and the application of hydrogen peroxide for the removal of toxic cyanobacteria from waste stabilization ponds. *Journal of Environmental Engineering, 137*(10), 952-960.

Bartram, J., & Chorus, I. (1999). *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*: CRC Press.

Bauzá, L., Aguilera, A., Echenique, R., Andrinolo, D., & Giannuzzi, L. (2014). Application of hydrogen peroxide to the control of eutrophic lake systems in laboratory assays. *Toxins, 6*(9), 2657-2675.

Beaver, J., & Havens, K. (1996). Seasonal and spatial variation in zooplankton community structure and their relation to possible controlling variables in Lake Okeechobee. *Freshwater Biology, 36*(1), 45-56.

Benndorf, J., Böing, W., Koop, J., & Neubauer, I. (2002). Top-down control of phytoplankton: the role of time scale, lake depth and trophic state. *Freshwater Biology, 47*(12), 2282-2295.

Best, J. H., Pflugmacher, S., Wiegand, C., Eddy, F. B., Metcalf, J. S., & Codd, G. A. (2002). Effects of enteric bacterial and cyanobacterial lipopolysaccharides, and of microcystin-LR, on glutathione S-transferase activities in zebra fish (Danio rerio). *Aquatic Toxicology, 60*(3-4), 223-231.

Brookes, J. D., & Carey, C. C. (2011). Resilience to blooms. *Science, 334*(6052), 46-47.

Buratti, F. M., Manganelli, M., Vichi, S., Stefanelli, M., Scardala, S., Testai, E., & Funari, E. (2017). Cyanotoxins: Producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. *Archives of Toxicology, 91*(3), 1049-1130.

Carmichael, W. (1992). Cyanobacteria secondary metabolites—the cyanotoxins. *Journal of applied bacteriology, 72*(6), 445-459.

Carmichael, W. (1997). The cyanotoxins. *Advances in botanical research, 27*, 211-257.

Chen, L., Chen, J., Zhang, X., & Xie, P. (2016). A review of reproductive toxicity of microcystins. *Journal of hazardous materials, 301*, 381-399.

Codd, G. A., BELL, S. G., Kaya, K., WARD, C. J., BEATTIE, K. A., & METCALF, J. S. (1999). Cyanobacterial toxins, exposure routes and human health. *European Journal of Phycology, 34*(4), 405-415.

Conley, D. J., Paerl, H. W., Howarth, R. W., Boesch, D. F., Seitzinger, S. P., Havens, K. E., . . . Likens, G. E. (2009). Controlling eutrophication: nitrogen and phosphorus. In: American Association for the Advancement of Science.

Cooper, W. J. (1994). Factors affecting the distribution of H_2O_2 in surface water. *Environmental chemistry of lakes and reservoirs*, 391-422.

Coveney, M., Stites, D., Lowe, E., Battoe, L., & Conrow, R. (2002). Nutrient removal from eutrophic lake water by wetland filtration. *Ecological Engineering, 19*(2), 141-159.

Daly, R. I., Ho, L., & Brookes, J. D. (2007). Effect of chlorination on Microcystis aeruginosa cell integrity and subsequent microcystin release and degradation. *Environmental Science & Technology, 41*(12), 4447-4453.

Davis, T. W., Berry, D. L., Boyer, G. L., & Gobler, C. J. (2009). The effects of temperature and nutrients on the growth and dynamics of toxic and non-toxic strains of Microcystis during cyanobacteria blooms. *Harmful Algae, 8*(5), 715-725.

Davis, T. W., & Gobler, C. J. (2016). Preface for Special Issue on "Global expansion of harmful cyanobacterial blooms: Diversity, ecology, causes, and controls". *Harmful Algae, 100*(54), 1-3.

Dia, S., Alameddine, I., & El-Fadel, M. (2019). Quantifying the efficacy of diquat dibromide in controlling Microcystis aeruginosa and Aphanizomenon flos-aquae in comparison to copper sulfate and potassium permanganate. *Environmental Science: Water Research & Technology, 5*(1), 140-151.

Dia, S., Alameddine, I., Salam, D., & El-Fadel, M. (2016). Controlling Microcystis aeruginosa blooms in a freshwater system: a comparative assessment across three chemical algaecides. In C. A. Brebbia (Ed.), *WIT Transactions on Ecology and the Environment* (Vol. 209, pp. 277). Wessex Institute, UK: WIT Press.

Doblin, M. A., Coyne, K. J., Rinta-Kanto, J. M., Wilhelm, S. W., & Dobbs, F. C. (2007). Dynamics and short-term survival of toxic cyanobacteria species in ballast water from NOBOB vessels transiting the Great Lakes—implications for HAB invasions. *Harmful Algae, 6*(4), 519-530.

Drabkova, M. (2007). Methods for control of the cyanobacterial blooms development in lakes. *Masaryk University: Research Centre for Environmental Chemistry and Ecotoxicology. Brno, Czech Republic*.

Dziga, D., Maksylewicz, A., Maroszek, M., & Marek, S. (2018). Combined treatment of toxic cyanobacteria Microcystis aeruginosa with hydrogen peroxide and microcystin biodegradation agents results in quick toxin elimination. *Acta Biochimica Polonica, 65*(1).

Dziga, D., Wasylewski, M., Wladyka, B., Nybom, S., & Meriluoto, J. (2013). Microbial degradation of microcystins. *Chemical research in toxicology, 26*(6), 841-852.

Eaton, A., & Franson, M. (2005). American Water Works Association & Water Environment Federation 2005 Standard Methods for the Examination of Water and Wastewater. *Washington, DC*.

Elder, J. F., & Horne, A. J. (1978). Copper cycles and CuSO 4 algicidal capacity in two California lakes. *Environmental management, 2*(1), 17-30.

Elliott, J. A. (2010). The seasonal sensitivity of cyanobacteria and other phytoplankton to changes in flushing rate and water temperature. *Global Change Biology, 16*(2), 864-876.

Fan, J., Daly, R., Hobson, P., Ho, L., & Brookes, J. (2013). Impact of potassium permanganate on cyanobacterial cell integrity and toxin release and degradation. *Chemosphere, 92*(5), 529-534.

Fan, J., Ho, L., Hobson, P., & Brookes, J. (2013). Evaluating the effectiveness of copper sulphate, chlorine, potassium permanganate, hydrogen peroxide and ozone on cyanobacterial cell integrity. *water research, 47*(14), 5153-5164.

Fan, J., Hobson, P., Ho, L., Daly, R., & Brookes, J. (2014). The effects of various control and water treatment processes on the membrane integrity and toxin fate of cyanobacteria. *Journal of hazardous materials, 264*, 313-322.

Fan, J., Rao, L., Chiu, Y.-T., & Lin, T.-F. (2016). Impact of chlorine on the cell integrity and toxin release and degradation of colonial Microcystis. *water research, 102*, 394-404.

Gao, L., Pan, X., Zhang, D., Mu, S., Lee, D.-J., & Halik, U. (2015). Extracellular polymeric substances buffer against the biocidal effect of H2O2 on the bloom-forming cyanobacterium Microcystis aeruginosa. *Water Research, 69*, 51-58.

Gulati, R. D., & Van Donk, E. (2002). Lakes in the Netherlands, their origin, eutrophication and restoration: state-of-the-art review. In *Ecological Restoration of Aquatic and Semi-Aquatic Ecosystems in the Netherlands (NW Europe)* (pp. 73-106): Springer.

Heisler, J., Glibert, P. M., Burkholder, J. M., Anderson, D. M., Cochlan, W., Dennison, W. C., . . . Humphries, E. (2008). Eutrophication and harmful algal blooms: a scientific consensus. *Harmful Algae, 8*(1), 3-13.

Ho, L., Hoefel, D., Palazot, S., Sawade, E., Newcombe, G., Saint, C. P., & Brookes, J. D. (2010). Investigations into the biodegradation of microcystin-LR in wastewaters. *Journal of hazardous materials, 180*(1-3), 628-633.

Ho, L., Lambling, P., Bustamante, H., Duker, P., & Newcombe, G. (2011). Application of powdered activated carbon for the adsorption of cylindrospermopsin and microcystin toxins from drinking water supplies. *water research, 45*(9), 2954-2964.

Hobson, P., Dickson, S., Burch, M., Thorne, O., Tsymbal, L., House, J., . . . Lin, T. (2012). Alternative and innovative methods for source water management of algae and cyanobacteria. *Water Research Foundation: Denver, CO, USA*.

Hoeger, S. J., Shaw, G., Hitzfeld, B. C., & Dietrich, D. R. (2004). Occurrence and elimination of cyanobacterial toxins in two Australian drinking water treatment plants. *Toxicon, 43*(6), 639-649.

Humphries, S., & Widjaja, F. (1979). A simple method for separating cells of Microcystis aeruginosa for counting. *British Phycological Journal, 14*(4), 313-316.

Huo, X., Chang, D.-W., Tseng, J.-H., Burch, M. D., & Lin, T.-F. (2015). Exposure of microcystis aeruginosa to hydrogen peroxide under light: Kinetic modeling of cell rupture and simultaneous microcystin degradation. *Environmental Science & Technology, 49*(9), 5502-5510.

IARC. (2010). Ingested nitrate and nitrite, and cyanobacterial peptide toxins. *IARC monographs on the evaluation of carcinogenic risks to humans, 94*.

Jankowiak, J., Hattenrath-Lehmann, T., Kramer, B. J., Ladds, M., & Gobler, C. J. (2019). Deciphering the effects of nitrogen, phosphorus, and temperature on cyanobacterial bloom intensification, diversity, and toxicity in western Lake Erie. *Limnology and Oceanography, 64*(3), 1347-1370.

Jones, G. J., & Orr, P. T. (1994). Release and degradation of microcystin following algicide treatment of a Microcystis aeruginosa bloom in a recreational lake, as determined by HPLC and protein phosphatase inhibition assay. *water research, 28*(4), 871-876.

Jurczak, T., Tarczynska, M., Izydorczyk, K., Mankiewicz, J., Zalewski, M., & Meriluoto, J. (2005). Elimination of microcystins by water treatment processes—examples from Sulejow Reservoir, Poland. *water research, 39*(11), 2394-2406.

Kansole, M. M., & Lin, T.-F. (2017). Impacts of Hydrogen Peroxide and Copper Sulfate on the Control of Microcystis aeruginosa and MC-LR and the Inhibition of MC-LR Degrading Bacterium Bacillus sp. *Water, 9*(4), 255.

Kehr, J. C., Zilliges, Y., Springer, A., Disney, M. D., Ratner, D. D., Bouchier, C., . . . Dittmann, E. (2006). A mannan binding lectin is involved in cell–cell attachment in a toxic strain of Microcystis aeruginosa. *Molecular microbiology, 59*(3), 893-906.

Kenefick, S., Hrudey, S., Peterson, H., & Prepas, E. (1993). Toxin release from Microcystis aeruginosa after chemical treatment. *Water Science and Technology, 27*(3-4), 433-440.

Khan, F. A., & Ansari, A. A. (2005). Eutrophication: an ecological vision. *The botanical review, 71*(4), 449-482.

Lewis, P. A., Klemm, D. J., Lazorchak, J. M., Norberg-King, T. J., Peltier, W. H., & Heber, M. A. (1994). *Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms*: US Environmental Protection Agency, Environmental Monitoring Systems Laboratory.

Li, J., Li, R., & Li, J. (2017). Current research scenario for microcystins biodegradation–a review on fundamental knowledge, application prospects and challenges. *Science of The Total Environment, 595*, 615-632.

Lin, T.-F., Chang, D.-W., Lien, S.-K., Tseng, Y.-S., Chiu, Y.-T., & Wang, Y.-S. (2009). Effect of chlorination on the cell integrity of two noxious cyanobacteria and their releases of odorants. *Journal of Water Supply: Research and Technology—AQUA, 58*(8), 539-551.

Lone, Y., Koiri, R. K., & Bhide, M. (2015). An overview of the toxic effect of potential human carcinogen Microcystin-LR on testis. *Toxicology Reports, 2*, 289-296.

Lürling, M., Meng, D., & Faassen, E. (2014). Effects of hydrogen peroxide and ultrasound on biomass reduction and toxin release in the cyanobacterium, Microcystis aeruginosa. Toxins 6, 3260–3280. In.

Ma, J., Brookes, J. D., Qin, B., Paerl, H. W., Gao, G., Wu, P., . . . Zhang, Y. (2014). Environmental factors controlling colony formation in blooms of the cyanobacteria Microcystis spp. in Lake Taihu, China. *Harmful Algae, 31*, 136-142.

Massey, I. Y., Yang, F., Ding, Z., Yang, S., Guo, J., Al-Osman, M., . . . Zeng, W. (2018). Exposure routes and health effects of microcystins on animals and humans: A mini-review. *Toxicon, 151*, 156-162.

Matthijs, H. C., Visser, P. M., Reeze, B., Meeuse, J., Slot, P. C., Wijn, G., . . . Huisman, J. (2012). Selective suppression of harmful cyanobacteria in an entire lake with hydrogen peroxide. *water research, 46*(5), 1460-1472.

McElhiney, J., & Lawton, L. A. (2005). Detection of the cyanobacterial hepatotoxins microcystins. *Toxicology and applied pharmacology, 203*(3), 219-230.

Michalak, A. M., Anderson, E. J., Beletsky, D., Boland, S., Bosch, N. S., Bridgeman, T. B., . . . Daloğlu, I. (2013). Record-setting algal bloom in Lake Erie caused by agricultural and meteorological trends consistent with expected future conditions. *Proceedings of the National Academy of Sciences, 110*(16), 6448-6452.

Mikula, P., Zezulka, S., Jancula, D., & Marsalek, B. (2012). Metabolic activity and membrane integrity changes in Microcystis aeruginosa–new findings on hydrogen peroxide toxicity in cyanobacteria. *European Journal of Phycology, 47*(3), 195-206.

Newcombe, G., House, J., Ho, L., Baker, P., & Burch, M. (2010). Management strategies for cyanobacteria (blue-green algae): A guide for water utilities. *Water Quality Research Australia (WQRA), Reserach Report, 74*.

Nicholson, B. C., Rositano, J., & Burch, M. D. (1994). Destruction of cyanobacterial peptide hepatotoxins by chlorine and chloramine. *water research, 28*(6), 1297-1303.

Paerl, H. W., Gardner, W. S., Havens, K. E., Joyner, A. R., McCarthy, M. J., Newell, S. E., . . . Scott, J. T. (2016). Mitigating cyanobacterial harmful algal blooms in aquatic ecosystems impacted by climate change and anthropogenic nutrients. *Harmful Algae, 54*, 213-222.

Paerl, H. W., & Otten, T. G. (2013). Harmful cyanobacterial blooms: causes, consequences, and controls. *Microbial ecology, 65*(4), 995-1010.

Paerl, H. W., & Scott, J. T. (2010a). Throwing fuel on the fire: synergistic effects of excessive nitrogen inputs and global warming on harmful algal blooms. *Environmental Science & Technology, 44*(20), 7756-7758.

Paerl, H. W., & Scott, J. T. (2010b). Throwing fuel on the fire: synergistic effects of excessive nitrogen inputs and global warming on harmful algal blooms. In: ACS Publications.

Pereira, S., Zille, A., Micheletti, E., Moradas-Ferreira, P., De Philippis, R., & Tamagnini, P. (2009). Complexity of cyanobacterial exopolysaccharides: composition, structures, inducing factors and putative genes involved in their biosynthesis and assembly. *FEMS microbiology reviews, 33*(5), 917-941.

Qian, H., Hu, B., Yu, S., Pan, X., Wu, T., & Fu, Z. (2012). The effects of hydrogen peroxide on the circadian rhythms of Microcystis aeruginosa. *PloS one, 7*(3).

Qian, H., Yu, S., Sun, Z., Xie, X., Liu, W., & Fu, Z. (2010). Effects of copper sulfate, hydrogen peroxide and N-phenyl-2-naphthylamine on oxidative stress and the expression of genes involved photosynthesis and microcystin disposition in Microcystis aeruginosa. *Aquatic Toxicology, 99*(3), 405-412.

Rastogi, R. P., Sinha, R. P., & Incharoensakdi, A. (2014). The cyanotoxin-microcystins: current overview. *Reviews in Environmental Science and Bio/Technology, 13*(2), 215-249.

Regel, R. H., Brookes, J. D., Ganf, G. G., & Griffiths, R. W. (2004). The influence of experimentally generated turbulence on the Mash01 unicellular Microcystis aeruginosa strain. *Hydrobiologia, 517*(1-3), 107-120.

Rice, E. W., Baird, R. B., Eaton, A. D., & Clesceri, L. S. (2012). *Standard methods for the examination of water and wastewater* (Vol. 10): American Public Health Association Washington, DC.

Smith, J. L., & Haney, J. F. (2006). Foodweb transfer, accumulation, and depuration of microcystins, a cyanobacterial toxin, in pumpkinseed sunfish (Lepomis gibbosus). *Toxicon, 48*(5), 580-589.

Spoof, L., Jaakkola, S., Važić, T., Häggqvist, K., Kirkkala, T., Ventelä, A.-M., . . . Meriluoto, J. (2020). Elimination of cyanobacteria and microcystins in irrigation water—effects of hydrogen peroxide treatment. *Environmental Science and Pollution Research*, 1-15.

Svrcek, C., & Smith, D. W. (2004). Cyanobacteria toxins and the current state of knowledge on water treatment options: a review. *Journal of Environmental Engineering and Science, 3*(3), 155-185.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M. F., Park, H.-D., . . . Yu, S.-Z. (1996). Detection of microcystins, a blue-green algal hepatotoxin, in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay.

USEPA. (1995). Method 551.1: Determination of Chlorination Disinfection Byproducts, Chlorinated Solvents, and Halogenated Pesticides/Herbicides in Drinking Water by Liquid–Liquid Extraction and Gas Chromatography With Electron-Capture Detection. *Methods for the Determination of Organic Compounds in Drinking Water: Supplement III*.

USEPA. (2002). Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. In: USEPA Washington.

Visser, P., Ibelings, B., Van Der Veer, B., Koedood, J., & Mur, R. (1996). Artificial mixing prevents nuisance blooms of the cyanobacterium Microcystis in Lake Nieuwe Meer, the Netherlands. *Freshwater Biology*, *36*(2), 435-450.

Wang, Q., Niu, Y., Xie, P., Chen, J., Ma, Z., Tao, M., . . . Guo, L. (2010). Factors affecting temporal and spatial variations of microcystins in Gonghu Bay of Lake Taihu, with potential risk of microcystin contamination to human health. *The Scientific World Journal*, *10*, 1795-1809.

Wang, Z., Li, D., Qin, H., & Li, Y. (2012). An integrated method for removal of harmful cyanobacterial blooms in eutrophic lakes. *Environmental Pollution*, *160*, 34-41.

Watanabe, M. F., & Oishi, S. (1985). Effects of environmental factors on toxicity of a cyanobacterium (Microcystis aeruginosa) under culture conditions. *Applied and Environmental microbiology*, *49*(5), 1342-1344.

WHO. (2003). Cyanobacterial toxins: microcystin-LR in drinking water. *Guidelines for Drinking Water Quality*, 2.

WHO, W. H. O. (2003). *Cyanobacterial Toxins: Microcystin-LR in Drinking-Water Background Document for Development of WHO Guidelines for Drinking-Water Quality. 2003* (Vol. 2).

Wu, Z.-x., Gan, N.-q., Huang, Q., & Song, L.-r. (2007). Response of Microcystis to copper stress–do phenotypes of Microcystis make a difference in stress tolerance? *Environmental Pollution*, *147*(2), 324-330.

Yang, Z., Kong, F., Shi, X., & Cao, H. (2006). Morphological response of Microcystis aeruginosa to grazing by different sorts of zooplankton. *Hydrobiologia*, *563*(1), 225-230.

Zimba, P., Khoo, L., Gaunt, P., Brittain, S., & Carmichael, W. (2001). Confirmation of catfish, Ictalurus punctatus (Rafinesque), mortality from Microcystis toxins. *Journal of Fish Diseases*, *24*(1), 41-47.

Appendix A

Sample collection and culturing

Collection

Experimental samples used in this study were taken from the shore of the Qaraoun Reservoir, 10 cm below the surface of the lake. Trip to the lake was made during algal bloom were *Microcystis* bloom were obtained mid-July 2019 as shown in the figure below (Figure A1). When arrived at the laboratory, the collected sample were observed under the microscope to ensure that the cells of the collected samples from this bloom were the targeted cyanobacteria, *Microcystis*.

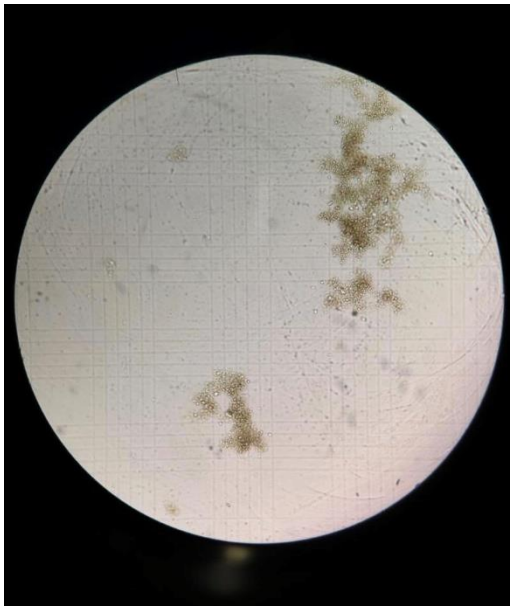


Figure A1 Microscopic observation of *Microcystis Aeruginosa* Colonies

Culturing

Collected samples were cultured in a 20 L beaker, that had air bubbled in it to ensure no carbon limitation and boost mixing. Water temperature was maintained at 25^{o}C, and a 12:12 light:dark cycle was implemented (Figure A2).

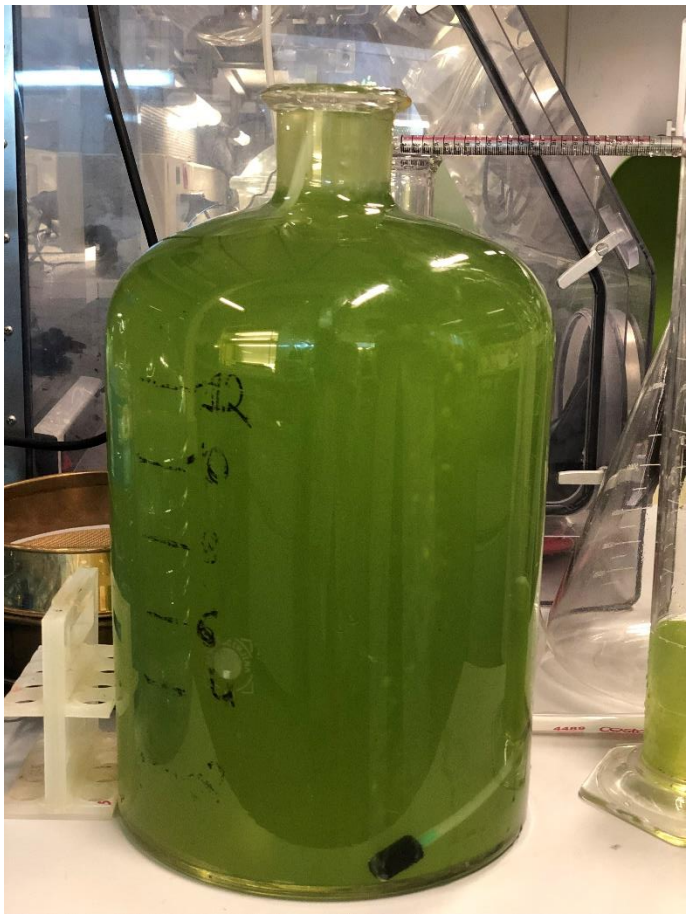


Figure A2 Culturing setup for cyanobacteria inhibition experiments

Samples that were culture were enriched with BG-11 medium (Sigma Aldrich, BG-11 Freshwater Solution, 50X). For culturing with BG-11, as recommended, for every 1 L of samples, 20 ml of BG-11 were added.

To secure the health of the algae with a mean density of $1x10^7$ cells/ml, samples were sub-cultured in 500 ml Erlenmeyer flasks, diluted with Milli-Q water and with the addition of the BG-11 medium, until an exponential phase was achieved. (Figure A3)

Figure A3 Sub-culturing setup with mean density $1x10^7$ cells/ml

Appendix B

Daily measurement of Temperature and pH

On the day that the samples were harvested from the lake, the ambient water temperature was measured at 28.9 ^{o}C and the pH of the lake was 8.87. Temperature and pH in the lab were measured on a daily basis throughout the experiment across all flasks. For each algaecide and for each dosage combination, the average values of the triplicate are reported in Table B1.

Table B1 Daily pH levels in the inhibition flasks

	Day 1		Day 2		Day 3		Day 4	
Algaecide	Temperature (^{o}C)	pH	Temperature (^{o}C)	pH	Temperature (^{o}C)	pH	Temperature (^{o}C)	pH
	pH and Temperature after algaecide treatment							
Control	26.53	7.18	27.63	7.22	25.63	7.34	26.07	7.81
H_2O_2 (1mg/L)	27.59	7.15	27.80	7.24	26.98	7.25	28.00	6.98
H_2O_2 (7mg/L)	27.23	7.18	27.55	7.49	26.38	7.50	27.58	7.35
H_2O_2 (10mg/L)	26.87	7.21	27.12	7.58	25.90	7.68	26.55	7.48
$CuSO_4$ (1mg/L)	26.99	6.95	27.42	7.21	25.40	7.14	26.53	7.01
	pH after chlorination treatment with 5mg/L							
H_2O_2 (1mg/L)		7.61		6.81		6.94		6.30
H_2O_2 (7mg/L)		7.88		7.69		6.98		7.42
H_2O_2 (10mg/L)		8.25		7.06		7.48		8.45
$CuSO_4$ (1mg/L)		7.54		7.29		7.14		6.37
	pH after chlorination treatment with 10mg/L							
H_2O_2 (1mg/L)		9.10		7.05		6.94		6.62
H_2O_2 (7mg/L)		8.27		7.97		7.18		7.15
H_2O_2 (10mg/L)		9.15		7.06		8.04		7.48
$CuSO_4$ (1mg/L)		8.47		7.31		7.15		6.63

Appendix C

Daily Cell Count

From each of the Erlenmeyer flasks, and on a daily basis, cells were counted. A sample of 0.1 ml of each flask was taken and placed on a hemocytometer slide and covered with a cover slip, using a Zeiss Fluorescence microscope (Axiovert 200). The technique of counting the cell through a hemocytometer was implemented from the Marienfeld counting chamber manual. Hence, in order to make cell counting simpler, an excel sheet was prepared based on the following formula:

$$\frac{Numer\ of\ cells}{Counted\ area\ (mm2) * Chamber\ depth\ (mm) * Dilution} = \frac{Cells}{ml}\ of\ Cyanobacteria$$

The average of the cell counts over the triplicated are shown in Table C1

Table C1 Average cell densities of Microcystis of each dosage of algaecide

	Day 1	Day 2	Day 3	Day 4
Control	1.16E+07	8.14E+06	1.27E+07	1.21E+07
H_2O_2 (1mg/L)	6.23E+06	4.06E+06	2.58E+06	2.70E+05
H_2O_2 (7mg/L)	4.29E+06	2.69E+06	1.40E+06	3.03E+05
H_2O_2 (10mg/L)	3.96E+06	2.81E+06	1.10E+06	1.58E+05
$CuSO_4$ (1mg/L)	3.71E+06	1.80E+06	1.06E+06	1.13E+05
H_2O_2 (1mg/L) + Cl_2 (5mg/L)	3.32E+06	1.39E+06	1.01E+06	7.83E+04
H_2O_2 (1mg/L) + Cl_2 (10mg/L)	2.06E+06	2.05E+06	1.06E+06	8.00E+04
H_2O_2 (7mg/L) + Cl_2 (5mg/L)	1.73E+06	1.72E+06	7.03E+05	2.42E+04
H_2O_2 (7mg/L) + Cl_2 (10mg/L)	1.32E+06	1.23E+06	7.09E+05	3.42E+04
H_2O_2 (10mg/L) + Cl_2 (5mg/L)	1.97E+06	9.85E+05	6.63E+05	5.00E+04
H_2O_2 (10mg/L) + Cl_2 (10mg/L)	1.94E+06	7.99E+05	6.74E+05	3.25E+04
$CuSO_4$ (1mg/L) + Cl_2 (5mg/L)	1.27E+06	1.03E+06	7.11E+05	2.00E+04
$CuSO_4$ (1mg/L) + Cl_2 (10mg/L)	1.16E+06	9.46E+05	6.80E+05	2.50E+04

Appendix D

Daily Microcystin Measurement

Microcystin concentrations were determined using the Enzyme Linked Immunosorbent Assay (ELISA) technique, provided by the USEPA (USEPA Method 546). To test the extracellular microcystin levels, samples were first filtered through a Whatman® GF/C glass microfiber filter, and immediately analyzed by the ELISA method. To test the total microcystin, about a volume of 2 ml of the sample was exposed to three freeze/thaw cycle before filtration to ensure that all cells have lysed and to release the toxins from the cells. The novel methodology used to fit a semi log curve, and to calculate the microcystin concentrations. Abraxis manual was followed as shown below.

Importance of Microcystins/Nodularins Determination

Most of the world's population relies on surface freshwaters as its primary source for drinking water. The drinking water industry is constantly challenged with surface water contaminants that must be removed to protect human health. Toxic cyanobacterial blooms are an emerging issue worldwide due to increased source water nutrient pollution caused by eutrophication. Microcystins and Nodularins are cyclic toxin peptides. Microcystins (of which there are many structural variants, or congeners) have been found in fresh water throughout the world. To date, approximately 80 variants of Microcystin have been isolated. The most common variant is Microcystin-LR. Other common Microcystin variants include YR, RR, and LW. These toxins are produced by many types of cyanobacteria (blue-green algae), including *Microcystis, Anabaena, Oscillatoria, Nostoc, Anabaenopsis,* and terrestrial *Hapalosiphon*. Nodularins are produced by the genus *Nodularia* and are found in marine and brackish water.

Acute poisoning of humans and animals constitutes the most obvious problem from toxic cyanobacterial blooms, and in several cases has lead to death. Human and animal exposure to these toxins occurs most frequently through ingestion of water, through drinking or during recreational activities in which water is swallowed. These toxins mediate their toxicity by inhibiting liver function and are potent inhibitors of the serine/threonine protein phosphatases, and therefore may act as tumor promoters.

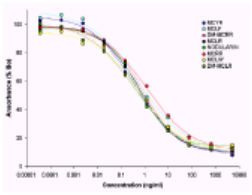
To protect consumers from adverse health effects caused by these toxins, the World Health Organization (WHO) has proposed a provisional upper limit for Microcystin-LR of 1.0 ppb (µg/L) in drinking water.

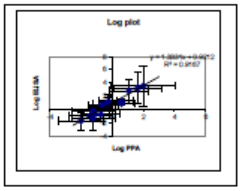
Performance Data

Test sensitivity: The detection limit for this assay, based on MC-LR, is 0.10 ppb (µg/L).

Test reproducibility: Coefficients of variation (CVs) for standards: <10%; for samples: <15%.

Selectivity*: The assay exhibits very good cross-reactivity with all cyanobacterial cyclic peptide toxin congeners tested to date (see cross-reactivity illustration below).





Samples: Sample correlation between HPLC, PPA, and ELISA methods showed a good correlation (see ELISA and PPA correlation above).

References

(1) W. J. Fischer, I. Garthwaite, C.O. Miles, K.M. Ross, J.B. Aggen, A.R. Chamberlin, N.A. Towers, and D.R. Dietrich, Congener-Independent Immunoassay for Microcystins and Nodularins." Environ. Sci. Technol. 35, 2001, 4849-4856.
(2) Worldwide Patenting PCT WO 01/18059 A2.
(3) U.S. Patent Number 6,967,240.
(4) U.S. Patent Number 9,739,777.

*QuikLyse™ reagents may be used in a method of U.S. Patent 9,739,777

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R062218OH

Microcystins-ADDA ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Congener-Independent* Determination of Microcystins and Nodularins in Water Samples

Product No. 520011OH

1. General Description

The Abraxis Microcystins-ADDA ELISA is an immunoassay for the quantitative and sensitive congener-independent* detection of Microcystins and Nodularins in water samples. This test is suitable for the quantitative and/or qualitative detection of Microcystins and Nodularins in water samples **[please refer to the appropriate technical bulletins for sample collection, handling, and treatment of drinking (treated and untreated) and recreational water samples]**. If necessary, positive samples can be confirmed by HPLC, protein phosphatase assay, or other conventional methods.

2. Safety Instructions

The standard solutions in the test kit contain small amounts of Microcystins. The substrate solution contains tetramethylbenzidine (TMB) and the stop solution contains diluted sulfuric acid. Avoid contact of the TMB and stopping solution with skin and mucous membranes. If these reagents come in contact with skin, wash with water.

3. Storage and Stability

The Microcystins-ADDA ELISA kit should be stored in the refrigerator (4–8°C). The solutions must be allowed to reach room temperature (20-25°C) before use. Reagents may be used until the expiration date on the box. Consult state, local, and federal regulations for proper disposal of all reagents.

4. Test Principle

The test is an indirect competitive ELISA for the congener-independent detection of Microcystins and Nodularins. It is based on the recognition of Microcystins, Nodularins, and their congeners by specific antibodies. Toxin, when present in a sample, and a Microcystins-protein analogue immobilized on the plate compete for the binding sites of the anti-Microcystins/Nodularins antibodies in solution. The plate is then washed and a second antibody-HRP label is added. After a second washing step and addition of the substrate solution, a color signal is generated. The intensity of the blue color is inversely proportional to the concentration of Microcystins present in the sample. The color reaction is stopped after a specified time and the color is evaluated using an ELISA reader. The concentrations of the samples are determined by interpolation using the standard curve constructed with each run.

5. Limitations of the Microcystins-ADDA ELISA, Possible Test Interference

Numerous organic and inorganic compounds commonly found in water samples have been tested and found not to interfere with this test. However, due to the high variability of compounds that may be found in water samples, test interferences caused by matrix effects cannot be completely excluded.

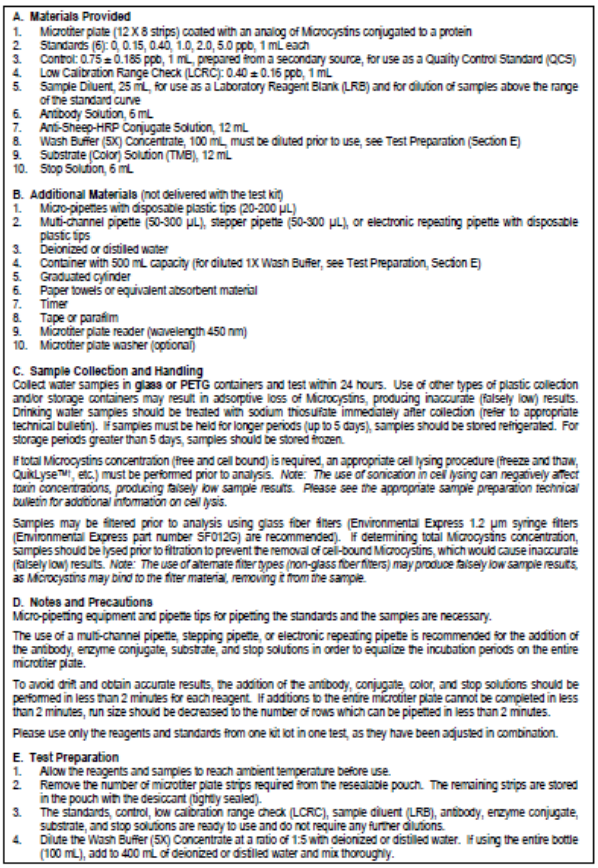
Samples containing methanol must be diluted to a concentration < 5% methanol to avoid matrix effects.

Seawater samples must be diluted to a concentration ≤ 2.5% to avoid matrix effects. Alternately, if a lower detection limit is required, interfering compounds can be removed from seawater or brackish water samples prior to analysis. Please see the Microcystins in Brackish Water or Seawater Sample Preparation for the Microcystins-ADDA ELISA Technical Bulletin (available upon request).

No matrix effects have been observed with samples which have been treated with sodium thiosulfate at concentrations ≤ 1 mg/mL.

Mistakes in handling the test can cause errors. Possible sources for such errors include: inadequate storage conditions of the test kit, incorrect pipetting sequence or inaccurate volumes of the reagents, too long or too short incubation times during the immune and/or substrate reaction, and extreme temperatures during the test performance (lower than 10°C or higher than 30°C).

As with any analytical technique (GC, HPLC, etc.), positive results requiring regulatory action should be confirmed by an alternative method.



A. Materials Provided

1. Microtiter plate (12 X 8 strips) coated with an analog of Microcystins conjugated to a protein
2. Standards (6): 0, 0.15, 0.40, 1.0, 2.0, 5.0 ppb, 1 mL each
3. Control: 0.75 ± 0.185 ppb, 1 mL, prepared from a secondary source, for use as a Quality Control Standard (QCS)
4. Low Calibration Range Check (LCRC): 0.40 ± 0.16 ppb, 1 mL
5. Sample Diluent, 25 mL, for use as a Laboratory Reagent Blank (LRB) and for dilution of samples above the range of the standard curve
6. Antibody Solution, 6 mL
7. Anti-Sheep-HRP Conjugate Solution, 12 mL
8. Wash Buffer (5X) Concentrate, 100 mL, must be diluted prior to use, see Test Preparation (Section E)
9. Substrate (Color) Solution (TMB), 12 mL
10. Stop Solution, 6 mL

B. Additional Materials (not delivered with the test kit)

1. Micro-pipettes with disposable plastic tips (20-200 µL)
2. Multi-channel pipette (50-300 µL), stepper pipette (50-300 µL), or electronic repeating pipette with disposable plastic tips
3. Deionized or distilled water
4. Container with 500 mL capacity (for diluted 1X Wash Buffer, see Test Preparation, Section E)
5. Graduated cylinder
6. Paper towels or equivalent absorbent material
7. Timer
8. Tape or parafilm
9. Microtiter plate reader (wavelength 450 nm)
10. Microtiter plate washer (optional)

C. Sample Collection and Handling

Collect water samples in **glass or PETG** containers and test within 24 hours. Use of other types of plastic collection and/or storage containers may result in adsorptive loss of Microcystins, producing inaccurate (falsely low) results. Drinking water samples should be treated with sodium thiosulfate immediately after collection (refer to appropriate technical bulletin). If samples must be held for longer periods (up to 5 days), samples should be stored refrigerated. For storage periods greater than 5 days, samples should be stored frozen.

If total Microcystins concentration (free and cell bound) is required, an appropriate cell lysing procedure (freeze and thaw, QuikLyse™, etc.) must be performed prior to analysis. *Note: The use of sonication in cell lysing can negatively affect toxin concentrations, producing falsely low sample results. Please see the appropriate sample preparation technical bulletin for additional information on cell lysis.*

Samples may be filtered prior to analysis using glass fiber filters (Environmental Express 1.2 µm syringe filters (Environmental Express part number SF012G) are recommended). If determining total Microcystins concentration, samples should be lysed prior to filtration to prevent the removal of cell-bound Microcystins, which would cause inaccurate (falsely low) results. *Note: The use of alternate filter types (non-glass fiber filters) may produce falsely low sample results, as Microcystins may bind to the filter material, removing it from the sample.*

D. Notes and Precautions

Micro-pipetting equipment and pipette tips for pipetting the standards and the samples are necessary.

The use of a multi-channel pipette, stepping pipette, or electronic repeating pipette is recommended for the addition of the antibody, enzyme conjugate, substrate, and stop solutions in order to equalize the incubation periods on the entire microtiter plate.

To avoid drift and obtain accurate results, the addition of the antibody, conjugate, color, and stop solutions should be performed in less than 2 minutes for each reagent. If additions to the entire microtiter plate cannot be completed in less than 2 minutes, run size should be decreased to the number of rows which can be pipetted in less than 2 minutes.

Please use only the reagents and standards from one kit lot in one test, as they have been adjusted in combination.

E. Test Preparation

1. Allow the reagents and samples to reach ambient temperature before use.
2. Remove the number of microtiter plate strips required from the resealable pouch. The remaining strips are stored in the pouch with the desiccant (tightly sealed).
3. The standards, control, low calibration range check (LCRC), sample diluent (LRB), antibody, enzyme conjugate, substrate, and stop solutions are ready to use and do not require any further dilutions.
4. Dilute the Wash Buffer (5X) Concentrate at a ratio of 1:5 with deionized or distilled water. If using the entire bottle (100 mL), add to 400 mL of deionized or distilled water and mix thoroughly.

F. Working Scheme

The microtiter plate consists of 12 strips of 8 wells, which can be used individually for the test. The standards must be run with each test. Never use the values of standards which have been determined in a test performed previously.

Std 0-Std5: Standards
Contr.: Control (QC5)
LCRC: Low Calibration Range Check
LRB: Laboratory Reagent Blank
Samp1, Samp2, etc: Samples

G. Assay Procedure

1. Add **50 µL of the standard solutions, control, LCRC, LRB, or samples** into the wells of the test strips according to the working scheme given. Analysis in duplicate or triplicate is recommended.
2. Add **50 µL of the antibody solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for **90 minutes** at room temperature.
3. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips **three times** using the diluted wash buffer. Please use at least a volume of **250 µL of 1X wash buffer** for each well and each washing step. ***Blot the inverted plate after each wash step*** on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
4. Add **100 µL of the enzyme conjugate solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for **30 minutes** at room temperature.
5. Remove the covering, decant the contents of the wells into a sink, and blot the inverted plate on a stack of paper towels. Wash the strips **three times** using the diluted wash buffer. Please use at least a volume of **250 µL of 1X wash buffer** for each well and each washing step. ***Blot the inverted plate after each wash step*** on a stack of paper towels. After the last wash/blot, check the wells for any remaining buffer in the wells, and if necessary, remove by additional blotting.
6. Add **100 µL of substrate (color) solution** to the individual wells successively using a multi-channel pipette or a stepping pipette. Cover the wells with parafilm or tape and mix the contents by moving the strip holder in a circular motion on the benchtop for 30 seconds. Be careful not to spill the contents. Incubate the strips for **20-30 minutes** at room temperature. Protect the strips from sunlight.
7. Add **50 µL of stop solution** to the wells in the same sequence as for the substrate (color) solution using a multi-channel pipette or a stepping pipette.
8. Read the absorbance at 450 nm using a microplate ELISA photometer within 15 minutes after the addition of the stopping solution.

H. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 4-Parameter (preferred) or Logit/Log. For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Microcystins concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control (QC5), LCRC, LRB, and samples will then yield levels in ppb of Microcystins by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Microcystins than standard 1 (0.15 ppb) should be reported as containing < 0.15 ppb of Microcystins. Samples showing a higher concentration than standard 5 (5.0 ppb) must be diluted to obtain accurate results. The concentration of the positive control (QC5) provided should be 0.75 ± 0.185 ppb; the LCRC should be 0.40 ± 0.16 ppb.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the standards. Samples with lower absorbances than a standard will have concentrations of Microcystins greater than that standard. Samples which have higher absorbances than a standard will have concentrations of Microcystins less than that standard.

Initial intracellular and total microcystin of the mother batch were measured at the beginning, their concentrations were 61.9 µg/L and 75.7 µg/L respectively. The average of the MC-LR concentrations after algaecide treatment, after filtration process, and after chlorination with both dosages (5 and 10 mg/L) over the triplicates are shown in Table D1

Table D1 Average MC-LR concentration in µg/L

	Day 1			Day 2			Day 3			Day 4		
	Intracellular	Extracellular	Total	Intracellular	Extracellular	Total	Intracellular	Extracellular	Total	Intracellular	Extracellular	Total
	MC-LR concentrations in µg/L after algaecide treatment											
Control	54.86	2.20	57.06	38.80	1.65	40.45	33.34	2.70	36.04	19.00	2.67	21.67
H_2O_2 (1mg/L)	34.23	4.33	38.56	23.78	1.90	25.68	13.81	2.67	16.48	13.83	2.25	16.15
H_2O_2 (7mg/L)	9.85	3.46	13.30	9.11	1.98	11.09	9.73	2.59	12.59	7.51	2.11	10.33
H_2O_2 (10mg/L)	6.19	4.53	11.01	5.74	2.16	7.90	5.59	2.63	8.22	3.11	2.46	5.57
$CuSO_4$ (1mg/L)	10.91	3.65	14.56	0.36	1.99	2.30	1.04	1.84	2.88	0.89	2.07	2.96
	MC-LR concentrations in µg/L after filtration treatment											
H_2O_2 (1mg/L)	38.80	1.65	40.45	33.34	2.70	9.81	5.52	2.45	7.98	5.06	2.06	7.12
H_2O_2 (7mg/L)	2.72	3.48	6.20	3.02	2.19	5.21	3.38	2.52	5.89	0.99	2.17	3.16
H_2O_2 (10mg/L)	0.97	4.16	5.13	2.68	2.29	4.97	1.12	2.70	3.82	0.85	2.15	3.00
$CuSO_4$ (1mg/L)	3.41	3.81	7.23	0.51	1.56	2.08	-0.49	2.20	1.71	-0.42	1.95	1.53
	MC-LR concentrations in µg/L after chlorination with 5 mg/L											
H_2O_2 (1mg/L)	38.80	1.65	40.45	33.34	2.70	4.48	0.56	1.97	2.54	1.49	3.98	5.48
H_2O_2 (7mg/L)	0.33	3.01	3.34	0.68	2.40	3.08	0.50	3.04	3.54	0.08	2.13	2.21
H_2O_2 (10mg/L)	0.46	3.19	3.65	1.39	2.47	3.86	0.58	2.96	3.54	0.16	2.62	2.78
$CuSO_4$ (1mg/L)	0.84	1.86	2.70	0.08	1.09	1.18	0.04	0.90	0.94	0.06	2.17	2.23
	MC-LR concentrations in µg/L after chlorination with 10 mg/L											
H_2O_2 (1mg/L)	0.20	2.41	2.61	0.34	4.16	4.50	0.27	1.91	2.18	0.50	4.63	5.14
H_2O_2 (7mg/L)	0.26	2.59	2.84	0.49	2.41	2.90	0.41	2.69	3.10	0.03	2.07	2.10
H_2O_2 (10mg/L)	0.28	3.18	3.45	0.43	1.98	2.40	0.34	2.77	3.11	0.08	2.21	2.29
$CuSO_4$ (1mg/L)	0.52	0.90	1.43	0.03	0.90	0.92	0.01	0.86	0.87	0.04	1.77	1.82