Release and Degradation of Microcystin-LR Following Exposures of Microcystis to Copper-Based Algaecides

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ABSTRACT

Microcystins (MCs) are hepatotoxic endotoxins produced by cyanobacteria such as *Microcystis* that can cause both ecological and human health risks, as well as economic losses in freshwater resources. The use of copper-based algaecides to manage MC-producing cyanobacteria is sometimes limited due to perceived risks of MC release from cyanobacterial cells and persistence of toxins in water resources. The aim of this research was to investigate the influence of copper concentration and form on MC release and persistence, as well as potential consequences of “no-action” management decisions (i.e. no treatment). To achieve these objectives, a tiered approach was employed utilizing both laboratory and field experiments and the commonly problematic microcystin-LR (MC-LR) producing cyanobacterium, *Microcystis*. In experiments that investigated MC release, a positive relationship was measured between copper concentrations and aqueous MC in both laboratory and field experiments. The positive relationship between copper concentration and aqueous MC-LR was observed at effective algaecide concentrations (i.e. concentrations that decreased cell density and chlorophyll a), demonstrating that lower copper concentrations can be as effective as relatively higher concentrations in controlling *Microcystis* while minimizing the proportion of MC-LR released from the cellular to aqueous phase. In experiments that investigated MC degradation, copper exposures influenced rates and extents of MC-degradation. However, these effects were concentration dependent and only occurred at copper exposures greater than concentrations registered for algaecidal use (i.e. 2-5 times greater than registered label concentrations [~1.0 mg Cu/L]). Influences of copper form on MC release and
degradation were tested in laboratory experiments, but did not significantly influence
either MC release or degradation rates. Total MC concentrations were also measured
throughout each experiment in untreated controls and compared against total MC
concentrations in copper treatments. Results indicated that total MC concentrations either
increased or remained stable in untreated controls throughout all experiments, compared
to copper treatments where MC concentrations typically decreased to background
(depending on exposure concentration); thus demonstrating the potential consequence of
“no-action” decisions. Results of these studies provide a more thorough understanding of
the influences of copper algaecide concentration and formulation on MC-LR release,
MC-LR degradation, and potential consequences of “no-action” management decisions.
These studies can be used to inform more accurate risk evaluations and use of copper-
based algaecides for management of MC-LR producing Microcystis.
DEDICATION

To my parents, Steve and Joyce Iwinski, for their unwavering support in me, and every goal I have endeavored to achieve; and to my fiancé, Brad, for his patient and steady support through 3 years, 1,127 miles, and daily research updates.
ACKNOWLEDGMENTS

I would first like to thank my advisor, Dr. John H. Rodgers, Jr., for his continuous guidance and support in me and my goals, and his steadfast commitment to education and science. I would also like to thank my advisory committee Dr. James Castle, Dr. Matt Huddleston, Dr. Alan Johnson, and Dr. Burton Suedel for the time and expertise they have shared with me. I would like to thank Lonza and the Midwest Aquatic Plant Management Society for funding this research. I would also like to thank Dr. Monique Haakensen, Dr. Vanessa Friesen, and Jenny Liang from Contango Strategies for their expertise, microbial analyses, and thorough manuscript reviews. I would also like to thank Dr. Wayne Chao for his analytical expertise and support.

I would like to thank my fellow graduate students, Ciera M. Kinley, Alyssa J. Calomeni, Tyler D. Geer, Andrew D. McQueen, and Maas Hendrikse. They were not only instrumental in the completion of this research but have left me with a lifetime of memories and lasting friendships. Last but not least, I want to thank my sisters, Seva and Virginia Iwinski, for their lifelong generosity and support.
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ORGANIZATION OF DISSERTATION

This dissertation consists of six chapters including the Introduction and Conclusion chapters. The body is comprised of four independent manuscripts formatted for publication in scientific journals. Therefore, some redundancy of material may be required.

CHAPTER II: Cellular and Aqueous Microcystin-LR Following Laboratory Exposures of Microcystis aeruginosa to Copper Algaecides

Published in Chemosphere (March 2016)

CHAPTER III: Comparison of Laboratory and Field Responses of the Microcystin Producing Cyanobacteria (Microcystis sp.) to Copper-Based Algaecide Exposures

In review (September 2016)

CHAPTER IV: Influence of Copper Algaecide Concentration and Formulation on Microcystin-LR Degradation

In review (September 2016)

CHAPTER V: Microcystin-LR Release and Degradation Following Exposures of M. aeruginosa to a Chelated Copper Algaecide

In review (November 2016)
CHAPTER ONE
Introduction

Toxins produced by cyanobacteria can result in ecological and human health risks, as well as economic losses in water resources (Carmichael 1989, 2001). Microcystins (MC) are hepatotoxic endotoxins produced by a number of cyanobacterial genera including *Microcystis*, *Planktothrix*, *Anabaena*, *Oscillatoria*, *Nostoc*, *Hapalosiphon*, and *Anabaenopsis* (Graham et al. 2008). Following exposure, MCs are acutely toxic to mammals, birds, and fish (Malbrouck and Kestemont 2006, WHO 2003). MC effects in organisms include liver damage (WHO 2003), tumor promotion (Falconer 1991), and MCs have been linked to neurological disorders such as Alzheimer’s and ALS (amyotrophic lateral sclerosis) in humans (Li et al. 2012). Economic losses due to the presence of MCs in water resources stem from tourism and property value declines due to lake closures, as well as management and treatment costs of the noxious algae or produced toxins (Carmichael 1989, 2001). There are over 140 structural variants (Westrick et al. 2010) of MCs and a provisional drinking water guideline and drinking water health advisory of 1 and 1.6 µg/L for total MC (intracellular + aqueous MC) have been established by the WHO and USEPA, respectively (WHO 2003, EPA 2015). The aforementioned guidelines and much research on MCs have focused on microcystin-LR, which is among the most toxic and frequently problematic congeners of MC (WHO 2003).
One management strategy for decreasing concentrations of MCs in aquatic systems is to decrease densities of MC-producing cyanobacteria using algaecides. However, use of algaecides, specifically copper-based algaecides, is sometimes limited due to perceived risks related to their application including release of intracellular MCs to surrounding waters, and persistence of MCs following release. These concerns are based on studies stating that loss of algal cell membrane integrity due to copper exposures results in release of intracellular MCs (e.g. Kenefick et al. 1993, Jones and Orr 1994, Touchette et al. 2008, Polyak et al. 2013, Zhou et al. 2013, Fan et al. 2013 and 2014, Greenfield et al. 2014, Tsai 2015), and that MC persistence may occur due to copper effects on MC-degrading bacterial populations (Jones and Orr 1994). Due to concerns regarding MC release and persistence, some states (i.e. Ohio, Michigan, Florida) have implemented regulations prohibiting copper applications, or requiring additional permits for treatment once potential MC-producers reach a specified cell density (i.e. >50,000 cells/mL; Ohio EPA’s General NPDES Permit No. OHG87000).

Based on available literature (Jones and Orr 1994), the widely accepted model (Figure 1.1) is that copper exposures cause MCs to release and persist, regardless of copper form and concentration. Field studies contributing to this model used relatively high (Touchette et al. 2008: ≥1ppm) or undisclosed (Kenefick et al. 1993, Jones and Orr 1994) concentrations of a single copper form. After examination of these studies, a number of additional hypotheses arise that could alter the current model. These hypotheses include the influence of copper concentration and form on both MC release as
well as MC degradation following copper algaecide exposures. Based on the concentration-dependent response of organisms to copper exposures (EPA 2007, Calomeni et al. 2014, Geer et al. 2016), it is reasonable to hypothesize that algal responses in terms of MC release, and bacterial responses in terms of MC degradation are also copper concentration dependent. In addition, form of constituents, including copper, may also result in altered exposures (i.e. chelated copper compounds [copper ethanolamines, copper citrates and gluconates] compared to copper salts [CuSO₄]; Murray-Gulde et al. 2002, Calomeni et al. 2014) and subsequently altered responses. Finally, potential consequences of “no action” decisions (i.e. increased or unchanging total MC concentrations) should also be considered, as risks of MCs in aquatic systems

Figure 1.1 Current model for MC following copper algaecide exposures
are not limited to aqueous forms of MCs and may be incurred from exposures to total MCs as well (WHO 2003, EPA 2015).

In order to investigate effects of copper concentrations, forms, and “no-action” decisions (i.e. cyanobacterial responses in untreated controls), a tiered approach was employed utilizing both laboratory and field experiments and the commonly problematic MC-producing cyanobacterium, *Microcystis*. Laboratory toxicity experiments were used to determine exposure-response relationships for target organism responses (Rand et al. 1995, Calomeni et al. 2014, Geer et al. 2016). Laboratory experiments facilitated testing a wide range of copper concentrations and formulations in relatively controlled and un-confounded settings. Laboratory experiments were performed with both cultured organisms and formulated water, and with field collected organisms and site water. This allowed for performance of controlled studies while still employing site-specific factors that may alter exposures and subsequent organism responses (i.e. water hardness, alkalinity, conductivity, pH, and organism sensitivity [i.e. naïve cultured algae versus field collected algae]).

In addition to laboratory experiments, field mesocosm experiments incorporated a wider range of environmental parameters (i.e. natural sunlight, temperature, sediment). Mesocosm studies, or “simulated field studies (SFSs)” are artificially bound systems or enclosures within a field site that are designed to simplify specific ecosystems (Graney et al. 1995). Although full-scale field studies are typically more realistic than SFSs, SFSs are more useful for prediction and measuring exposure-response relationships by
providing adequate replication and improving the ability to manipulate treatments (Graney et al. 1995). SFSs are used to measure copper concentration and form-dependent MC release and persistence following Microcystis exposure to copper algaecides. SFSs provide more realistic experiments under field conditions while maintaining comparable cell densities within and among treatments, inhibit migration of algae through wind and currents, and afford adequate replication for statistical comparison. Convergence of data from SFSs and laboratory experiments can increase the value of information used to inform the current MC model and advise regulatory and management decisions for MC-producing cyanobacteria.

The overall objective of this research was to answer specific questions regarding a number of widely accepted, yet not well understood or documented risks involving MCs following the use of copper-based algaecides. The specific questions and objectives posed in the four individual studies that comprise this body of research are listed below. This dissertation consists of six chapters including the Introduction and Conclusion chapters. The four independent body chapters are formatted for publication in scientific journals. Therefore, some redundancy of material may be required.
Chapter Two Objectives

CELLULAR AND AQUEOUS MICROCYSTIN-LR FOLLOWING LABORATORY EXPOSURES OF *MICROCYSTIS AERUGINOSA* TO COPPER ALGAECIDES

The objectives of this study were: 1) to measure relationships between copper exposure concentrations and responses of *M. aeruginosa* in terms of total, cell bound, and aqueous MC-LR concentrations, and chlorophyll a; 2) compare responses of untreated *M. aeruginosa* to copper exposed algae over time (5 days) in terms of total MC-LR; and 3) to compare the differences between two copper formulations, copper sulfate (CuSO₄) and the chelated copper compound Cutrine-Plus® (copper-ethanolamine), by measuring responses of *M. aeruginosa* in terms of total, cell bound, and aqueous MC-LR concentrations, and chlorophyll a.

Chapter Three Objectives

COMPARISON OF LABORATORY AND FIELD RESPONSES OF THE MICROCYSTIN PRODUCING CYANOBACTERIA (*MICROCYSTIS*) TO COPPER-BASED ALGAECIDE EXPOSURES

The overall objective of this study was to use a tiered approach (i.e. laboratory toxicity experiment, laboratory toxicity experiment with field-collected algae, and a simulated field study) to measure and confirm the relationship between MC-LR<sub>Aq</sub> and copper concentrations following exposure of *M. aeruginosa* to a copper-based algaecide. Specific objectives were to measure and compare *M. aeruginosa* responses to copper algaecide exposures in laboratory and field experiments in terms of: (1) relationships between MC-LR<sub>Aq</sub> and copper concentration (i.e. effective concentration for 50% of
population \([EC_{50s}]\) and potency slopes), (2) release of \(MC-LR_{Aq}\) (i.e. percent \(MC-LR_{Aq}\) released from \(MC-LR_{Tot}\)), and (3) algal viability (i.e. chlorophyll \(a\) and cell density).

**Chapter Four Objectives**

INFLUENCE OF COPPER ALGAECIDE CONCENTRATION AND FORMULATION ON MICROCYSTIN-LR DEGRADATION

The overall objective of this laboratory study was to determine the influence of copper concentrations and formulations on degradation of \(MC-LR\) following copper algaecide exposures. The specific objectives were to 1) measure rates and extents of \(MC-LR_{Tot}\) degradation over time following exposures to a series of concentrations of three copper-based algaecides; 2) compare rates and extents of \(MC-LR\) degradation between copper concentrations (0, 0.1, 0.5, 1.0, and 5.0 mg Cu/L); 3) compare rates and extents of \(MC-LR\) degradation between copper formulations (\(CuSO_4\), Cutrine Plus\(^{®}\), and Algimycin\(^{®}\)); and 4) measure and compare relative abundance and diversity of bacteria among treatments (formulation and concentration).

**Chapter Five Objectives**

MICROCYSTIN-LR RELEASE AND DEGRADATION FOLLOWING EXPOSURES OF \(MICROCYSTIS\) TO A CHELATED COPPER ALGAECIDE

The overall objective of this simulated field (mesocosm) study was to determine the influence of copper concentrations on \(MC-LR\) degradation following copper algaecide exposures. The specific objectives in support of the overall objective were to 1)
measure responses of *M. aeruginosa* to targeted concentrations of Cutrine-Plus® in terms of chlorophyll *a* and cell density, 2) measure and compare concentrations of MC-LR$_{Tot}$ and MC-LR$_{Aq}$ following exposure to a series of copper concentrations, 3) measure and compare rates and extent of MC-LR$_{Tot}$ and MC-LR$_{Aq}$ degradation over time following exposure to a series of copper concentrations, and 4) measure and compare bacterial density, relative abundance, and diversity following exposure to a series of copper concentrations.
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chemical algaecides on the cell numbers and toxin content of the cyanobacteria *Microcystis aeruginosa* and *Anabaenopsis sp.* Environmental Management, 54: 1110-1120.


CHAPTER 2
CELLULAR AND AQUEOUS MICROCYSTIN-LR FOLLOWING LABORATORY EXPOSURES OF MICROCYSTIS AERUGINOSA TO COPPER ALGAECIDES

Abstract

Release of microcystin from algal cells influences use of copper-algaecides in water resources. Accurate data regarding relationships between copper-algaecide exposures and responses of microcystin-producing algae are needed to make informed management decisions. Responses of Microcystis aeruginosa were measured in terms of cellular microcystin-LR (MC-LR), aqueous MC-LR, and chlorophyll-a following exposure to concentrations of CuSO$_4$ and copper-ethanolamine. Comparisons were made between treated and untreated samples, and copper formulations. EC50s and slopes for _M. aeruginosa_ responses to copper exposures were calculated. Algal responses followed a sigmoidal exposure-response relationship, and cellular MC-LR and chlorophyll-a were negatively related to copper concentrations. Aqueous MC-LR increased with copper concentrations, although the increase in aqueous MC-LR was not proportional to decreases in cellular MC-LR and chlorophyll-a. Cellular MC-LR and chlorophyll a declined at a greater rate than aqueous MC-LR increased. Total MC-LR was less than untreated controls following copper exposure. Differences between copper formulations in terms of aqueous and total MC-LR concentrations were measured at concentrations of 0.5 and 1.0 mg Cu/L. Aqueous and total MC-LR were greater (10-20%) following exposure to CuSO$_4$ compared to copper-ethanolamine one day following exposure. The
positive relationship between copper concentration and aqueous MC-LR at 0.07-1.0mg Cu/L demonstrates that lower copper concentrations were as effective as higher concentrations in controlling *M. aeruginosa* while decreasing the total amount of MC-LR, and minimizing the proportion of MC-LR released to the aqueous-phase. Results serve to support more accurate risk evaluations of MC-LR concentrations when *M. aeruginosa* is exposed to copper-algaecides and when it is untreated.
Introduction

Toxins produced by noxious algae can interfere with use of freshwater resources as well as present risks to aquatic fauna, livestock, domestic pets, and humans. Microcystins (MCs) are common algal toxins produced by a number of Cyanobacteria genera including Anabaena, Anabaenopsis, Hapalosiphon, Microcystis, Nostoc, Oscillatoria, and Planktothrix. MCs are cyclic hepatotoxins that cause liver damage (WHO 2003), promote tumor production (Falconer 1991), and have been linked to neurological disorders such as Alzheimer’s and amyotrophic lateral sclerosis (ALS) (Li et al. 2012). There are over 80 structural variants of MC (Westrick et al. 2010) which are characterized by two variable amino acids in the MC structure that contribute to the compounds hydrophilicity, polarity and toxicity (WHO 2003). MC-LR (microcystin-leucine, arginine) (Table 2.1) is one of the most commonly problematic and toxic variants of MC, and much of MC research has been conducted on MC-LR (WHO 2003). A provisional drinking water guideline of 1 µg/L for total MC-LR has also been adopted by the World Health Organization (WHO 2003). Although MC-LR is not currently regulated in the United States, it has been listed on the United States Environmental Protection Agency’s (USEPA) Candidate Contaminant list for drinking water, and the USEPA has issued a health advisory for consumption of MC-LR in drinking water of 1.6 µg/L for adults and 0.3 µg/L for infants and young children (USEPA 2015). When algal densities and toxin concentrations interfere with use of water resources, management strategies, often involving chemical algaeicide treatment, are implemented.
Copper-based algaecides have been used for decades and remain one of the most effective algaecide treatments available to date. However, use of copper-based algaecides is sometimes limited due to perceived risks related to their application. One frequent concern involves the potential release or “leaking” of cell-bound MC following copper algaecide exposure. This concern is based on studies stating that loss of algal cell membrane integrity due to exposures of copper-based algaecides results in release of cell-bound MCs into surrounding waters (e.g. Kenefick et al. 1993, Jones and Orr 1994, Touchette et al. 2008, Polyak et al. 2013, Zhou et al. 2013, Fan et al. 2013 and 2014, Greenfield et al. 2014, Tsai 2015).

Based on these studies, the current widely accepted concept is that copper exposure causes MC to release, regardless of algaecide form and concentration. Field studies contributing to this hypothesis used relatively high (Touchette et al. 2008: ≥1ppm) or undisclosed (Kenefick et al. 1993, Jones and Orr 1994) copper concentrations. After examination of these studies a number of questions arise. The first of these is what relationship exists between copper exposure concentrations and aqueous and cellular MC concentrations? Algal responses such as cell density, photosynthesis, and cell membrane integrity demonstrate a sigmoidal exposure-response relationship following exposure to copper algaecides (Calomeni and Rodgers 2014). Studies that incorporated more than one copper exposure concentration such as Fan et al. (2013), Fan et al. (2014), Zhou et al. (2013), and Tsai (2015) found incremental increases in extracellular MC concentrations with increasing copper sulfate concentrations. Although some evidence of a relationship
between MC release and copper concentration exists from previous studies, it was not specifically an objective of the studies and was not thoroughly investigated or discussed. Confirmation of a relationship between MC release and copper exposure concentration may be accomplished using a larger range of concentrations, derived exposure-response relationships, and calculated EC$_{50}$s. This information may inform the current concept used to describe and predict MC release from the cellular to aqueous phase following copper exposure.

Another question arises regarding potential risks of aqueous MC compared to total MC. It is commonly stated or implied that aqueous MC is a greater ecological and human health risk compared to cellular MC. Aqueous MC may be a greater risk in drinking water sources when treatment facilities are not equipped with adequate filtration or oxidation to remove aqueous toxins (USEPA 2012, AWWA 2015). However, the WHO MC-LR guideline for human consumption was established for total MC-LR which includes both cell bound toxin and aqueous toxin (WHO 2003). Total MC is also a risk for domestic pets, livestock, and contact recreation as MC is released from ingested algal cells inside the stomach during digestion (Beasley et al. 1989). MC concentrations in water are generally positively correlated with algal cell density (Chorus and Bartram 2000, Zohary and Pais Madeira 1990). Therefore, allowing MC producing algae to grow unmanaged can result in increased total MC and consequently increased risk. Comparisons between total MC concentrations following treatment with copper-based
algaecides compared to total MC concentrations from untreated algae may provide important data for resource managers seeking to mitigate risks associated with total MC.

Lastly, the majority of studies of post-treatment MC release from exposures of copper algaecides used copper sulfate (CuSO$_4$) (Kenefick et al. 1993, Fan et al. 2013, Fan et al. 2014, Zhou et al. 2013). Algae may respond differently depending on the copper algaecide formulation they are exposed to, such as those chelated with ethanolamine or citrate and gluconate (Murry-Gulde et al. 2002, Calomeni et al. 2014). Copper sulfate is a copper salt while Cutrine-Plus® is copper chelated with ethanolamine (Table 2.2). Hypothetically, the two copper sources (CuSO$_4$ and Cutrine-Plus®) will dissociate into Cu$^{2+}$ and ethanolamine chelated copper compounds respectively. However for algae, chelated copper compounds are often more potent than CuSO$_4$ (Murray-Gulde et al. 2002, Calomeni et al. 2014). Previous studies have found that metals are transported more readily across cell membranes when complexed with ligands (Campbell et al. 2002). These differences may ultimately change the exposure to the algal cell and result in an altered response. It is important to explore these hypotheses before making or accepting sweeping predictions about the effects of all copper based algaecides and responses of microcystin producing algal species.

The objectives of this study were: 1) measure relationships between copper exposure concentrations and responses of *Microcystis aeruginosa* in terms of total, cell bound, and aqueous MC-LR concentrations, and chlorophyll *a*; 2) compare responses of untreated *M. aeruginosa* to copper-exposed algae over time (5 days) in terms of total
MC-LR; and 3) compare the differences between two copper formulations, copper sulfate (CuSO₄) and the chelated copper compound Cutrine-Plus® (copper-ethanolamine), by measuring responses of *M. aeruginosa* in terms of total, cell bound, and aqueous MC-LR concentrations, and chlorophyll *a*. 
Materials and Methods

Algal Culture

*M. aeruginosa* (CPCC 300) was selected for use in this study because it is a consistent and reliable producer of MC-LR. *M. aeruginosa* was cultured in ~35 liter aquaria in BG-11 medium. Water characteristics of BG-11 medium (Grobbelaar 2004) with 4.5x10⁶ cells of *M. aeruginosa* mL were: 1) pH = 8.55, 2) dissolved O₂ = 10.72 mg/L, 3) conductivity = 2,162 µs, 4) alkalinity = 9.2 mg CaCO₃, 5) hardness = 27 mg CaCO₃. Temperature was maintained at 21°C with an 18:6 light:dark cycle and light intensity of 1980-3340 LUX provided by cool-white fluorescent bulbs (Residential Ecolux 40 W, GE). Algae were maintained under these temperature and light conditions for the duration of the study. *M. aeruginosa* was obtained from the Canadian Phycological Culture Center (CPCC) at the University of Waterloo in Waterloo, Ontario, Canada.

Algaecide Exposures

A copper salt, (CuSO₄•5H₂O; Thermo Fisher Scientific, Inc.), and a chelated copper compound, Cutrine-Plus® (Applied Biochemists, Alpharetta, GA) chelated with ethanolamine, were selected for this study (Table 2.2). *M. aeruginosa* was exposed to a series of concentrations of these algaecides. 1,000 mg Cu/L stock solutions were prepared from CuSO₄ and Cutrine-Plus® using Nanopure® water before initiation of toxicity experiments. *M. aeruginosa* in 200 mL BG-11 medium was placed into separate 250 mL acid washed borosilicate beakers.
To measure the relationships between copper exposure concentrations and total, cellular, and aqueous MC-LR and chlorophyll \( a \), appropriate amounts of stock copper solutions were added creating seven concentrations, 0.02, 0.05, 0.07, 0.1, 0.2, 0.5 and 1.0 mg Cu/L as CuSO\(_4\) and Cutrine-Plus\textsuperscript{®} for \textit{M. aeruginosa}. Untreated controls did not receive copper additions. Acid soluble copper concentrations were measured at the initiation of the experiment to confirm exposures using an Agilent PSD 120 Flame Atomic Absorption Spectrometer (APHA 2005).

\textbf{Algal Response}

Responses of \textit{M. aeruginosa} to CuSO\(_4\) and Cutrine Plus\textsuperscript{®} were measured 1-5 days after treatment (1-5 DAT). \textit{M. aeruginosa} were pipetted from beakers for MC-LR and chlorophyll \( a \) concentration analysis. Beakers were not mixed prior to sampling as \textit{M. aeruginosa} is a gas vacuolated species and can maintain its buoyancy in the water column. Mixing may also have resulted in inclusion of cells in viability measures that had been adversely affected by copper and had settled out of the water column. Slopes and EC\textsubscript{50}s were calculated using measured copper concentrations for \textit{M. aeruginosa} response in terms of cellular MC-LR decrease and aqueous MC-LR increase 1 and 4 DAT. Slopes and EC\textsubscript{50}s for decreases in chlorophyll \( a \) were calculated 4 DAT only due to no measurable response 1 DAT. Percent error between measured and nominal copper exposure was 2-20\% for CuSO\(_4\) and Cutrine Plus\textsuperscript{®}. 

20
**Microcystin-LR Quantification**

MC-LR concentrations were measured using high performance liquid chromatography (HPLC) with ultraviolet detection. The following HPLC parameters were selected from the methods presented by Sangolkar et al. (2006): A Dionex Ultimate 3000® HPLC was utilized with an Acclaim Polar Advantage II® column (5µm, 120Å, 4.6x250nm); settings for analysis were: detection = 243 nm, temperature = 30°C, pressure = 1500 psi, flow = 1 mL/min, and injection volume = 60 µL; solvents included Nanopure® H₂O/ 0.1% trifluoroacetic acid and HPLC grade acetonitrile. Peak area was measured at a detention time of 14.0-14.5 minutes and MC-LR concentrations were calculated by comparing measured area units to known MC-LR standards (Beagle Bioproducts®, Columbus, Ohio). Known 100 µg /L MC-LR standards were measured between every 20, samples and variability was calculated to confirm measurement accuracy. Method specific detection limits for MC-LR (lowest concentration where the measured MC-LR concentration was significantly different from the measured solvent blank) (APHA 2005), using the 10-fold concentration factor described below, was 1.0 µg MC-LR/L (ANOVA, α=0.05, p=0.0022).

**Microcystin-LR Extraction and Concentration**

Prior to HPLC quantification, MC-LR was extracted and concentrated from samples. Subsamples of algae in each exposure were collected prior to exposure and on days 1, 2, 3, 4, and 5 following copper exposures. Extracellular MC-LR was separated by pipetting 10 mL of sample from each beaker and filtering through a Millipore®
0.45µm nitrocellulose filter. Filtrate was then concentrated by evaporation for approximately 8-12 hours. Once dry, concentrate was re-suspended in 1 mL of 80:20 Nanopure® water and HPLC grade acetonitrile, vortexed to mix, and transferred to 2 mL HPLC vials for HPLC quantification.

Cellular MC-LR was extracted by pipetting 10 mL of sample from each beaker and centrifuged at 3,300 rpm for 10 minutes. Supernatant was discarded and the algal pellet was then frozen at -80°C, thawed, evaporated, and re-suspended in 1 mL of 80:20 Nanopure® water and HPLC grade acetonitrile. Re-suspended pellet was stored at 4°C overnight (approximately 12 hours) in solvent and then sonicated using a Branson® 5210 sonic bath for 5 minutes. Samples were then centrifuged for 10 minutes at 3,300 rpm and the supernatant was transferred to 2 mL HPLC vials for HPLC quantification. Total MC-LR was determined by adding measured cellular MC-LR to measured aqueous microcystin-LR: Cell bound MC-LR + Aqueous MC-LR = Total MC-LR.

**Cell Viability: Chlorophyll a**

Subsamples of algae in each exposure were taken prior to exposure and then on days 1, 2, 3, 4, 5 and 7 following copper exposure. Chlorophyll a was measured using a modified spectrophotometric method (APHA 2005) on a SpectraMax M2 spectrofluorometer (Molecular Devices Corp, Sunnyvale, CA). The APHA (2005) method was modified by using Millipore® Nitrocellulose Membrane filters (Millipore®, Tullagreen, Ireland) rather than glass fiber filters. The use of membrane filters in substitution of glass fiber filters does not significantly affect chlorophyll a concentration.
estimates for planktonic algae (Moran et al. 1999). Chlorophyll a standards were acquired from Sigma Aldrich (C6144).

**Statistical Analysis**

Responses of cell bound MC-LR, aqueous MC-LR, and chlorophyll a in relation to copper exposure concentration were analyzed using non-linear regression with a sigmoid, 4P logistic fit function (JMP v.11). Inflection points calculated are synonymous with EC\textsubscript{50} values. Calculated EC\textsubscript{50} values and slopes were used to compare *M. aeruginosa* response measures and algaecide formulations. Differences between MC-LR concentrations in the control compared to MC-LR concentrations in copper exposures were measured using ANOVA (JMP v.11). ANOVA and Tukey’s pairwise test were also used to discern differences in *M. aeruginosa* in terms of chlorophyll a, total, cellular, and aqueous MC-LR between algaecides. Exposure-response graphs were created using GraphPad Prism (2011). A non-linear, sigmoidal 4P model was fit to the data to generate figures.
Results

*M. aeruginosa Responses to Copper Exposures*

Maximum responses of *M. aeruginosa* (where response measures plateaued and no longer changed with increased copper concentrations) in terms of cellular MC-LR and chlorophyll *a* occurred between 0.07 and 0.1 mg Cu/L, for both CuSO₄ and Cutrine Plus® (Figure 2.1). Chlorophyll *a* decreased by approximately 80% 4 DAT from pretreatment concentrations (510 µg chlorophyll *a* /L) for both CuSO₄ (162±30 µg chlorophyll *a* /L 4 DAT) and Cutrine Plus® (161±27 µg chlorophyll *a* /L 4 DAT). Cellular MC-LR decreased an order of magnitude from 51.2 µg/L MC-LR to less than 3 µg/L MC-LR 4 DAT for both CuSO₄ and Cutrine Plus®. Chlorophyll *a* continued to decrease over time and decreased to 77.1 µg/L by 7 DAT. Maximum response of *M. aeruginosa* in terms of aqueous MC-LR (maximum release of MC-LR to aqueous phase) occurred between 0.2 and 0.5 mg Cu/L 1 DAT (Figure 2.1). Maximum concentrations of aqueous MC-LR (µg/L) were 50.5±1.4 and 40.3±1.4 one day after exposure to CuSO₄ and Cutrine Plus® respectively (Figure 2.1, Table 2.4).

Cellular MC-LR and chlorophyll *a* were negatively related to copper concentrations, and aqueous MC-LR was positively related with copper concentrations (Figure 2.1, Table 2.3). Rate of change in response is defined as change in the response parameter (MC-LR or chlorophyll *a*) with change in copper concentration and is presented as the natural log of the response (y) over copper concentration (x) (Table 2.3). The rates of decrease of cellular MC-LR and chlorophyll *a* were significantly greater than
the rates of increase in aqueous MC-LR on 1 and 4 DAT (Table 2.3, Figure 2.1). That is, greater concentrations of copper were required to increase aqueous MC-LR than were required to decrease cellular MC-LR. Effective concentrations at which half of the maximum response was observed are presented as EC50s. EC50s 1 DAT for cellular and aqueous MC-LR were also not directly proportional; the effective copper concentration that resulted in a 50% decrease in cellular MC-LR was approximately 58 and 67% less than the copper concentration that resulted in a 50% increase in aqueous MC-LR for CuSO4 and Cutrine Plus® respectively (Table 2.3).

**Total MC-LR**

*Comparison Between Untreated Control and Copper Exposures*

Total MC-LR concentrations in the untreated control increased by 183% relative to pre-treatment concentrations, from 53±6 (µg/L) 1 DAT to 130±6 (µg/L) 5 DAT. Total MC-LR concentrations in copper treatments of 0.05-1.0 mg Cu/L were significantly less than the untreated control on days 2-5 of the study (α=0.05, p=<0.0001-0.0406) (Figure 2.3, Table 2.4). Following copper exposures of 0.07-1.0 mg Cu/L, no increases in total MC-LR were measured throughout the study (Table 2.4, Figure 2.2). Total MC-LR significantly decreased by 4 DAT (α=0.05, p=0.0001-0.0371) from the initial total MC-LR concentration (45.94 µg/L) following exposure to 0.07 and 0.1 mg Cu/L as both CuSO4 and Cutrine Plus® (Table 4). Four DAT, total MC-LR decreased by 65% and 75% at copper exposures of 0.07 mg Cu/L as CuSO4 and Cutrine Plus® respectively; and by 83% and 95% at 0.1 mg Cu/L for CuSO4 and Cutrine Plus® respectively (Figure 2.2).
Total MC-LR decreased 1 DAT by a maximum of 41% and 68% at 0.7 mg Cu/L for CuSO₄ and Cutrine Plus® respectively. Total MC-LR was significantly less than the control following exposure to copper concentrations of 0.05-0.07 for both CuSO₄ (31.3-38.1 µg MC-LR/L, α=0.05, p=<0.005-0.04) and Cutrine Plus® (19.3-35.0 µg MC-LR/L, α=0.05, p=<0.0001-0.004) (Figure 2.3, Table 2.4).

**Comparison of Algaecide Formulations**

No differences between algaecide formulations were measured in terms of EC₅₀s and slopes for aqueous MC-LR and chlorophyll a 1 and 4 DAT after exposure (Table 2.4). There were significant differences in the EC₅₀s for responses in cellular MC-LR 1 DAT, when Cutrine Plus® EC₅₀ [35 (50, 66) µg Cu/L] was less than CuSO₄ [58 (22, 46) µg Cu/L] (Table 2.4). Differences were measured between the two algaecide formulations in terms of aqueous, cellular, and total MC-LR 1 and 4 DAT (Table 2.4). Aqueous MC-LR concentrations were significantly greater following exposure to CuSO₄ compared to Cutrine Plus® at exposure concentrations of 0.5 mg Cu/L (α=0.05, p=0.0066, 0.0028) on 1 and 4 DAT, respectively, and at 1.0 mg Cu/L (α=0.05, p=0.047, 0.0005) 1 and 4 DAT, respectively. Total MC-LR was significantly greater following exposure to CuSO₄ compared to Cutrine Plus® at exposure concentrations of 0.5 mg Cu/L (α=0.05, p=0.0163, 0.0013) 1 and 4 DAT, respectively, and 1.0 mg Cu/L (α=0.05, p=0.0464, 0.0005) 1 and 4 DAT, respectively (Table 2.4).
Discussion

*M. aeruginosa Responses to Copper Exposure*

Changes in cellular and aqueous MC-LR depended on the exposure concentrations of copper for both CuSO₄ and Cutrine Plus®. Aqueous MC-LR concentrations were positively related to copper concentrations, and the amount of microcystin released to the aqueous phase increased with copper concentration. Increases in aqueous MC-LR concentrations with increasing copper concentration were also reported by Fan et al. (2013), Zhou et al. (2013) and Tsai (2015). At all copper concentrations that effectively decreased chlorophyll a concentrations in *M. aeruginosa* (0.07-1.0 mg Cu/L), a portion of the total MC-LR was released to the aqueous phase. However, the extent of release depended on copper concentrations where only 23.6/14.9% and 61.1/53.2% (CuSO₄/Cutrine Plus® %) were released to the aqueous phase following exposures to 0.07 and 0.1 mg Cu/L respectively, compared to 97.9/79.8% and 96.7/77.5% (CuSO₄/Cutrine Plus® %) at 0.5 and 1.0 mg Cu/L. The positive relationship between copper concentration and aqueous MC-LR at these effective algaecide concentrations (0.07-1.0 mg Cu/L) demonstrates that lower copper concentrations were as effective as higher concentrations in controlling *M. aeruginosa* while decreasing the amount of MC-LR released from cells to aqueous phase.

Release of MC-LR following copper exposure is likely due to loss of cell membrane integrity (Fan et al. 2014, Zhou et al. 2013). Copper toxicity to cells is possible through multiple modes of action including inhibition of cell functions due to
copper binding to non-copper proteins (Stevenson et al. 2013), disruption of photosynthetic processes (Qian et al. 2010, Jancula and Marsalek 2011), and production of reactive oxygen species (ROS) (Qian et al. 2010) due to the oxidation-reduction of free copper ions in the cell (Stevenson et al. 2013). Loss of cell membrane integrity is likely the result of ROS production within the cell, which is hypothesized to occur when copper influx exceeds efflux in the presence of high copper concentrations (Stevenson et al. 2013). This may explain why increases in copper concentrations resulted in greater release of MC-LR while chlorophyll a responses remained constant across a range of comparable copper exposures.

Increases in aqueous MC-LR concentrations were not directly proportional to decreases in cellular MC-LR concentrations. Theoretically, MC-LR will exist in either the cellular or aqueous phase and therefore if cellular MC-LR decreases, aqueous MC-LR will increase at the same rate and extent. This trend however was not observed in this study. To achieve 50% release of MC-LR to the aqueous phase, 50-100% more copper was required than that needed to decrease cellular MC-LR by the same extent. This disproportion between cellular and aqueous MC-LR responses is likely responsible for decreased total MC-LR at 0.05-0.1 mg Cu/L exposures 1 DAT. The MC-LR should have been accounted for in either the cellular or aqueous phase; however, the total MC-LR was approximately 50% less than higher (0.2-1.0 mg Cu/L) and lower copper exposures (Control-0.02 mg Cu/L). Reported aerobic degradation half-lives for MC-LR are typically between 4-14 days (Edwards et al. 2008), therefore it is unlikely that
degradation of released MC-LR 1 DAT was responsible for the decreased total MC-LR concentration. Another possibility is that the unaccounted for MC-LR was confined within cells that had settled following copper exposure. Due to the ability of *M. aeruginosa* to control its buoyancy, beakers were not mixed prior to sampling and settled cells were not analyzed.

In addition to differences between copper exposures in terms of total MC-LR concentrations measured 1 DAT, changes in total MC-LR concentrations over time were also dependent on copper concentration. Total MC-LR in the control increased by 183% while total MC-LR concentrations at effective copper exposures (0.07-1.0 mg Cu/L) either remained the same or decreased following exposure to 0.07-1.0 mg Cu/L in comparison to the untreated control. Following exposure to the maximum concentration of 1.0 mg Cu/L, MC-LR was present primarily in the aqueous phase and decreased by only 2/17% (CuSO₄/Cutrine Plus® %) by the end of the study period. Concentrations of total MC-LR following exposure to 0.1 mg Cu/L decreased by 83/95% (CuSO₄/Cutrine Plus® %) by the end of the study. Copper exposures at all effective concentrations were successful at either decreasing or preventing the increase in total MC-LR concentration in comparison to untreated samples where total MC-LR increased. This may have implications for increased risks in terms of total MC-LR when no mitigation technique is used and algae continue to grow unmanaged.

Although means for total and aqueous MC-LR were typically lower following exposure to Cutrine Plus® compared to CuSO₄, significant differences were only
measureable at copper exposures of 0.5 and 1.0 mg Cu/L. This may have been due to
greater variability in *M. aeruginosa* responses at copper exposures of 0.07-0.2 mg Cu/L.
Differences in responses of a single algae species to different copper algaecide
formulations have been reported (Calomeni et al. 2014). There were differences in EC$_{50}$s
for *M. aeruginosa* responses in terms of cellular MC-LR in this study; however, the
differences were only measurable 1 DAT. By 4 DAT, EC$_{50}$s for CuSO$_4$ and Cutrine
Plus$^\text{®}$ were not different.
Conclusions

Release of cellular MC-LR to the aqueous phase was positively correlated with copper concentrations. Both CuSO$_4$ and Cutrine Plus$^\circledR$ were effective at decreasing *M. aeruginosa*, as indicated by chlorophyll *a*, at concentrations of 0.07-1.0 mg Cu/L. Lower copper concentrations elicited responses in *M. aeruginosa* as effectively as higher concentrations while causing less MC-LR to release to the aqueous phase. Decreases in aqueous MC-LR concentrations after exposure were dependent on copper concentration. MC-LR concentrations decreased to a greater extent at lower copper exposure concentrations. However, all copper exposures between 0.07-1.0 mg Cu/L decreased or prevented the increase of total MC-LR. The untreated control continued to increase in total MC-LR concentrations, more than doubling by the end of the study.

The results of this study emphasize that copper concentration is an important factor in responses of *M. aeruginosa* in terms of MC-LR release. The positive relationship between copper concentration and aqueous MC-LR at the effective algaecide concentrations of 0.07-1.0 mg Cu/L demonstrates that lower copper concentrations were as effective as higher concentrations in controlling *M. aeruginosa* while decreasing the total amount of MC-LR compared to the control, and minimizing the proportion of MC-LR released from the cellular to aqueous phase. In addition, total MC-LR concentrations in control samples continued to increase over time, which may have implications for risks of a “no-action” decision in terms of total-MC-LR. These data serve to support more
accurate risk evaluations of total MC-LR concentrations when *M. aeruginosa* is exposed to copper algaeicides and when the alga is left untreated.
References


batch experiments. Archives of Environmental Contamination and Toxicology, 52: 489-495.


Zhou, S. Y. Shao, N. Gao, Y. Deng, J. Qiao, H. Ou, J. Deng. 2013. Effects of different algaeicides on the photosynthetic capacity, cell integrity, and microcystin-LR

Table 2.1. Physical, chemical, and biological characteristics of MC-LR.

<table>
<thead>
<tr>
<th>Microcystin-LR (L – leucine, R – arginine)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Formula</strong></td>
</tr>
<tr>
<td><strong>Molecular Weight</strong></td>
</tr>
<tr>
<td><strong>CAS Number</strong></td>
</tr>
<tr>
<td><strong>Color/ Form</strong></td>
</tr>
<tr>
<td><strong>Photolysis (half-life)</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Hydrolysis</strong></td>
</tr>
<tr>
<td><strong>Microbial Aerobic Degradation</strong> (half-life)</td>
</tr>
<tr>
<td><strong>Solubility in Water</strong></td>
</tr>
<tr>
<td><strong>Boiling point (°C)</strong></td>
</tr>
<tr>
<td><strong>Log(_{Kow})</strong></td>
</tr>
<tr>
<td><strong>Sorption Extent (%)</strong></td>
</tr>
<tr>
<td><strong>K(_d) (L kg(^{-1})) (Sediment)</strong></td>
</tr>
</tbody>
</table>

\(^a\)Tsuji et al. 1994  
\(^b\)Tsuji et al. 1995  
\(^c\)Edwards et al. 2008, WHO 2003  
\(^d\)Chen et al. 2008  
\(^e\)Rivasseau et al. 1998  
\(^f\)Wu et al. 2011  
\(^g\)Miller et al. 2001  
\(^h\)Morris et al. 2000  
\(^i\)Calculated from Munusamy et al. 2012 (Fig. 2, pg. 2395)  
\(^j\)Mohamed et al. 2007
Table 2.2 Characteristics of copper sulfate pentahydrate (CuSO₄) and Cutrine-Plus®.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CuSO₄•5H₂O</th>
<th>Cutrine®-Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active ingredient</td>
<td>Copper</td>
<td>Copper</td>
</tr>
<tr>
<td>% Active ingredient</td>
<td>23.7</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximum label concentration as copper</td>
<td>2 mg/L&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1 mg/L&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formulation</td>
<td>Copper sulfate pentahydrate</td>
<td>Copper ethanolamine complex&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Appearance</td>
<td>Blue crystals</td>
<td>Blue liquid</td>
</tr>
<tr>
<td>Water solubility</td>
<td>415,997 mg/L</td>
<td>Miscible&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Boiling point (°C)</td>
<td>106&lt;sup&gt;**&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific gravity (g/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.21&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.22</td>
</tr>
<tr>
<td>pH</td>
<td>3.22&lt;sup&gt;**&lt;/sup&gt;</td>
<td>10.3-10.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Physical and chemical characteristics are of the original compound unless otherwise noted.

* Cutrine<sup>®</sup>-Plus can mix with water in all proportions.

** Physical and chemical characteristics of saturated solution

<sup>a</sup> Applied Biochemists product label

<sup>b</sup> Applied Biochemists MSDS

<sup>c</sup> Murray-Gulde et al. 2002

<sup>e</sup> Chem One Ltd. product label
### Table 2.3 M. aeruginosa responses to copper exposures: EC$_{50}$s, Slopes, and R$^2$

<table>
<thead>
<tr>
<th></th>
<th>CuSO$_4$ 1 DAT</th>
<th>Cutrine Plus® 1 DAT</th>
<th>CuSO$_4$ 4 DAT</th>
<th>Cutrine Plus® 4 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cellular</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>58</td>
<td>35</td>
<td>52</td>
<td>52</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(50, 66)</td>
<td>(22, 46)</td>
<td>(49, 55)</td>
<td>(49, 55)</td>
</tr>
<tr>
<td>Slope</td>
<td>-0.06</td>
<td>-0.08</td>
<td>-0.08</td>
<td>-0.09</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(-0.08, -0.03)</td>
<td>(-0.1, -0.03)</td>
<td>(-0.1, -0.06)</td>
<td>(-0.1, -0.06)</td>
</tr>
<tr>
<td>R$^2$</td>
<td>0.96</td>
<td>0.90</td>
<td>0.99</td>
<td>0.98</td>
</tr>
<tr>
<td><strong>Aqueous</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>107</td>
<td>105</td>
<td>181</td>
<td>162**</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(84, 131)</td>
<td>(91, 119)</td>
<td>(151, 211)</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.03</td>
<td>0.04</td>
<td>0.03</td>
<td>**</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>(0.01, 0.05)</td>
<td>(0.02, 0.06)</td>
<td>(0.001, 0.05)</td>
<td></td>
</tr>
<tr>
<td>R$^2$</td>
<td>0.86</td>
<td>0.92</td>
<td>0.89</td>
<td>0.72</td>
</tr>
<tr>
<td><strong>Chlorophyll</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EC$_{50}$</td>
<td>*</td>
<td>*</td>
<td>60</td>
<td>50</td>
</tr>
<tr>
<td>a (95% CI)</td>
<td>*</td>
<td>*</td>
<td>(53, 66)</td>
<td>(40, 60)</td>
</tr>
<tr>
<td>Slope</td>
<td>*</td>
<td>*</td>
<td>-0.09</td>
<td>-0.07</td>
</tr>
<tr>
<td>(95% CI)</td>
<td>*</td>
<td>*</td>
<td>(-0.1, -0.04)</td>
<td>(-0.1, -0.03)</td>
</tr>
<tr>
<td>R$^2$</td>
<td>*</td>
<td>*</td>
<td>0.98</td>
<td>0.96</td>
</tr>
</tbody>
</table>

EC$_{50}$ values are presented as µg Cu/L as the specified alagecide. Slopes are presented as the natural log of the response (i.e. microcystin concentration; y)/exposure concentration (x). R$^2$ values represent goodness of fit of a sigmoidal, 4P logistic model for the corresponding exposure-response data. *represents no discernable response for that parameter on the specified day. **Data did not fit a sigmoidal curve (using the 4P logistic fit model used for all data), the EC$_{50}$ (median copper concentration between minimum and maximum aqueous MC-LR concentrations) was estimated based on this model but the confidence interval and slope could not be calculated.
Table 2.4 Total and Aqueous MC-LR concentrations following copper exposure

<table>
<thead>
<tr>
<th>Copper (µg/L)</th>
<th>Total MC-LR (µg/L) 1 DAT</th>
<th>Total MC-LR (µg/L) 4 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CuSO₄</td>
<td>Cutrine Plus®</td>
</tr>
<tr>
<td>Control±SE</td>
<td>53.1±5.9</td>
<td>53.1±5.4</td>
</tr>
<tr>
<td>0.02±SD</td>
<td>50.7±1.4</td>
<td>42.0±10.9</td>
</tr>
<tr>
<td>0.05±SD</td>
<td>36.0±16.1</td>
<td>20.1±10.7</td>
</tr>
<tr>
<td>0.07±SD</td>
<td>31.3±12.7</td>
<td>16.7±8.8</td>
</tr>
<tr>
<td>0.1±SD</td>
<td>38.1±4.4</td>
<td>32.4±2.5</td>
</tr>
<tr>
<td>0.2±SD</td>
<td>43.7±7.4</td>
<td>43.6±5.3</td>
</tr>
<tr>
<td>0.5±SD</td>
<td>56.1±3.2</td>
<td>46.0±3.1</td>
</tr>
<tr>
<td>1.0±SD</td>
<td>55.9±1.3</td>
<td>46.2±1.6</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Aqueous MC-LR (µg/L) 1 DAT</th>
<th>Aqueous MC-LR (µg/L) 4 DAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control±SD</td>
<td>2.4±1.0</td>
</tr>
<tr>
<td>0.02±SD</td>
<td>1.8±0.4</td>
</tr>
<tr>
<td>0.05±SD</td>
<td>8.9±8.9</td>
</tr>
<tr>
<td>0.07±SD</td>
<td>12.2±12.2</td>
</tr>
<tr>
<td>0.1±SD</td>
<td>31.5±4.1</td>
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<tr>
<td>0.2±SD</td>
<td>36.3±9.3</td>
</tr>
<tr>
<td>0.5±SD</td>
<td>50.5±2.2</td>
</tr>
<tr>
<td>1.0±SD</td>
<td>49.9±2.0</td>
</tr>
</tbody>
</table>

SD=standard deviation
Figure 2.1 Responses of *M. aeruginosa* after exposure to CuSO₄ and Cutrine Plus® in terms of cellular MC-LR and aqueous MC-LR (A) 1 and (B) 4 DAT and in terms of chlorophyll *a* (C) 4 DAT. Error bars = standard deviation. n=3 (per copper concentration)
Figure 2.2 Total MC-LR concentration on days 1-5 after treatment (DAT) with CuSO₄ and Cutrine Plus® at each copper exposure concentration and in the untreated control. Error bars = standard deviation
Figure 2.3 Cellular, aqueous, and total MC-LR concentrations for CuSO₄ and Cutrine Plus® 1 DAT. Error bars= standard deviation. n=3
CHAPTER 3

COMPARISON OF LABORATORY AND FIELD RESPONSES OF THE MICROCYSTIN PRODUCING CYANOBACTERIA (MICROCYSTIS) TO COPPER-BASED ALGAECIDE EXPOSURES

Abstract

Recent laboratory experiments with copper-based algaecides and microcystin-LR (MC-LR) producing Microcystis indicated that decreasing copper-exposure concentrations minimizes release of aqueous-MC-LR (MC-LR$_{Aq}$) while effectively decreasing Microcystis cell density and chlorophyll a concentrations. This study utilized a tiered approach to confirm exposure-response relationships between MC-LR$_{Aq}$ and copper concentration. Microcystis responses to copper-exposures were compared between laboratory and field experiments in terms of: (1) relationships between MC-LR$_{Aq}$ and copper concentration, (2) release of MC-LR$_{Aq}$, and (3) algal viability. Positive sigmoidal relationships between MC-LR$_{Aq}$ and copper concentration were measured in all experiments. Proportions of MC-LR$_{Aq}$ to MC-LR$_{Tot}$ were significantly less following exposure to minimum effective copper concentrations (0.07-0.3 mg Cu/L) compared to highest copper exposures (0.8-1.0 mg Cu/L). Cultured M. aeruginosa in laboratory experiments was more sensitive to copper exposures than field collected algae, as indicated by both chlorophyll a and cell density, and MC-LR$_{Aq}$ release. Field collected Microcystis responded similarly in laboratory and field experiments in terms of algal viability and MC-LR release. The incremental release of MC-LR from Microcystis within a range of effective copper concentrations highlights that using copper more efficiently
(i.e. utilizing the minimum concentration needed for control of *Microcystis*) decreases not only the amount of copper-algaecide required, but can also minimize MC-LR release. Analogous responses of *Microcystis* in laboratory and field experiments to similar copper-exposures emphasize the utility of preliminary laboratory experiments for predicting field responses. Presented results and the utilized approach are useful for informing management of risks associated with both MC-LRAq and MC-LRTot.
Introduction

Toxins produced by noxious algae can result in ecological and human health risks, as well as economic losses in water resources (Carmichael 1989, 2001). Ecological risks include exposures of aquatic and terrestrial organisms to toxins, and human health risks occur when people are exposed to toxins in potable and recreation waters. Economic losses stem from declining tourism and property values due to lake closures, as well as costs associated with management and treatment of noxious algae or produced toxins (Carmichael 1989, 2001). Microcystin (MC) is a potent hepatotoxin responsible for the majority of algal-related human and animal poisonings worldwide (WHO 2003). Microcystin-LR (MC-LR) is among the most toxic and commonly problematic congeners of MC, with the majority of MC research to date performed on MC-LR (WHO 2003). Although not all cyanobacteria are capable of MC-LR production, when cyanobacteria are actively producing MC-LR, MC-LR concentrations are frequently correlated with cell density (Graham et al. 2008) as MC-LR is contained within algal cells until cell lysis and death (Zohary and Pais Madeira 1990, Watanabe et al. 1992, Chorus and Bartram 2000). When rapid restoration of water resource uses is required (i.e. days to weeks), chemical treatments such as copper-based algaecides are often utilized. However, use of copper-based algaecides to manage MC-LR-producing algae is sometimes limited due to concerns regarding MC-LR release from algal cells following treatment (Kenefick et al. 1993, Jones and Orr 1994, Touchette et al. 2008, Polyak et al. 2013, Zhou et al. 2013, Fan et al. 2013 and 2014, Greenfield et al. 2014). Some states have implemented
regulations preventing copper applications, or requiring additional permits for treatment once potential MC-producers reach a specified cell density (i.e. >50,000 cells/mL; Ohio EPA’s General NPDES Permit No. OHG87000). The current focus on aqueous MC-LR (MC-LR_{Aq}) concentrations is likely based on an inaccurate indicator of risk. Concerns regarding MC-LR_{Aq} following chemical treatment may result in a “no-action” approach for MC-LR-producing algae. Untreated algal populations can continue to grow over time, increasing risks due to total MC-LR (MC-LR_{Aq} + MC-LR_{Cell} = MC-LR_{Tot}) as MC-LR concentrations typically increase with cell density (Tsai et al. 2015, Iwinski et al. 2016).

Potential risks incurred from both action and inaction emphasize the need for thorough investigation of available management strategies for MC-LR-producing algae. A better understanding of relationships between copper exposures and responses of MC-LR-producing algae in terms of MC-LR release can provide insight for more effective use of copper-based algaecides (i.e. maintained or improved efficacy and decreased risk). Recent laboratory experiments with copper-based algaecides and the MC-LR-producing cyanobacterium *Microcystis aeruginosa* suggest that decreasing copper exposure concentrations can decrease release of MC-LR_{Aq} while effectively controlling *M. aeruginosa* (Iwinski et al. 2016). Measured relationships between copper concentrations and MC-LR_{Aq}, within a range of copper concentrations that effectively controlled the problematic algae, achieved decreased MC-LR_{Aq} with decreasing copper concentration. This information could be directly applicable to management of MC-LR-producing algae with copper-based algaecides. In order to improve the utility of this information, it should
be corroborated with other lines of evidence (i.e. field studies). The aim of this research was to confirm exposure-response relationships between copper exposure concentrations and MC-LR release by comparing both laboratory and field studies in which MC-LR-producing *Microcystis* were exposed to a range of copper-based algaecide concentrations.

In this study a tiered approach was used to investigate copper concentration dependent MC-LR release from *Microcystis*. Three separate experiments (laboratory toxicity experiment with cultured *M. aeruginosa*, laboratory toxicity experiment with field-collected algae, and a simulated field study) exposed MC-LR-producing *Microcystis* to a chelated copper-based algaecide (Cutrine-Plus®). Laboratory studies are often the first step for determining responses of organisms and potential risks of chemical exposures. Laboratory toxicity experiments are used to determine exposure-response relationships for both target and non-target organisms to pesticides (Rand et al. 1995, Calomeni et al. 2015, Geer et al. [in review]). Laboratory studies provide un-confounded situations where exposures and responses are carefully controlled and measured. Realism can be increased in laboratory toxicity experiments by using field collected organisms and media (Graney et al. 1995). This allows for performance of controlled studies while still employing site-specific factors that may alter exposures and subsequent organism responses (i.e. water hardness, alkalinity, conductivity, pH, and organism sensitivity [i.e. naïve cultured versus field collected]). Although laboratory experiments with field-collected media are more representative of field water characteristics and organisms, they are conducted under optimized light and temperature conditions. Therefore, a higher tier
for validation of exposure-response relationships can be achieved by performing field experiments as exposures are conducted with more realistic environmental parameters (i.e. natural sunlight, temperature, sediment).

Simulated field studies (SFSs) are artificially bound systems or enclosures within a field site designed to simplify specific ecosystems (Graney et al. 1995). Although full-scale field studies are typically more realistic than SFSs, SFSs are more useful for prediction and measuring exposure-response relationships by providing adequate replication and improving the ability to manipulate and control treatment variables (Graney et al. 1995). SFSs are frequently a step in a tiered approach along with laboratory toxicity studies to validate exposure-response relationships or endpoints for a specific chemical and organism(s) (Graney et al. 1995). Using SFSs to measure concentration dependent MC-LR release from Microcystis provides a more realistic experiment under field conditions while maintaining comparable cell densities within and among treatments, inhibiting migration of algae through wind and currents, and affords adequate replication for statistical comparisons. Utilizing SFSs can support data measured in laboratory experiments illustrating relationships between MC-LR release and copper exposure concentration. A convergence of data from SFSs and laboratory experiments can increase the value of information that can be utilized by water resource managers when both MC-LR_{Aq} and MC-LR_{Tot} are concerns.

The overall objective of this study was to use a weight of evidence approach (i.e. laboratory toxicity experiment, laboratory toxicity experiment with field-collected algae,
and a simulated field study) to measure and confirm the relationship between MC-LRAq and copper concentrations following exposure of *Microcystis* to a copper-based algaecide. Specific objectives were to measure and compare *Microcystis* responses to copper algaecide exposures in laboratory and field experiments in terms of: (1) relationships between MC-LRAq and copper concentration (i.e. effective concentration for 50% of population [EC50s] and slopes), (2) release of MC-LRAq (i.e. percent MC-LRAq released from MC-LRTot), and (3) algal viability (i.e. chlorophyll a and cell density).
Materials and Methods

Experimental Conditions

Three separate experiments were conducted in this study, two laboratory experiments and one SFS utilizing ~50 L enclosures. The initial laboratory experiment was conducted with laboratory cultured *M. aeruginosa* in formulated media, and the second using field collected *Microcystis* and field water. Both laboratory experiments were conducted under similar experimental conditions (i.e. light and temperature; Table 3.1). Cultured *M. aeruginosa* strain CPCC 300 obtained from the Canadian Phycological Culture Center (CPCC) at the University of Waterloo in Waterloo, Ontario, Canada, was selected for use in the laboratory portion of this study because it is a consistent and reliable producer of MC-LR. CPCC 300 was cultured in ~35 L aquaria in BG-11 medium (Grobbelaar 2004). Water characteristics of BG-11 media containing *M. aeruginosa* are listed in Table 3.1. Field collected *Microcystis* was obtained from a small pond in Anderson, South Carolina. Cultured and field collected *Microcystis* were maintained under the same light and temperature conditions for the duration of the study. Laboratory exposures of cultured and field collected *Microcystis* were not performed simultaneously. Experiments with cultured *M. aeruginosa* were conducted December 2014 to January 2015 and experiments with field collected *Microcystis* were conducted in June of 2015. SFSs with *Microcystis* were conducted in September of 2015. Experimental conditions measured during the field portion of this study are listed in Table 3.1.
Field Site and Enclosures

SFSs were conducted in a small pond with a surface area of approximately 530 m² (0.13 acres), and an average depth of approximately 1.2 m (4 ft). The pond was located at TRI Environmental, Inc.’s Denver Downs Research Facility in Anderson, SC. In addition to in situ SFSs at the site, water and algae were collected from this facility and transported to Clemson University for use in laboratory experiments with field algae and water. The pond received both agricultural and livestock runoff from the surrounding 900-acre farm. Nutrient and element concentrations within the pond were determined by ICP at the Clemson University Agricultural Services Laboratory. Measured concentrations are expressed as follows in mg/L: P=0.5, K=38.4, Ca=21.7, Mg=8.9, Zn=<0.01, Cu=0.014, Mn=0.2, Fe=2.8, S=4.6, Na=5.5, B=0.03, Cl=14.6, NO₃=0.7.

High-density polyethylene, 30-gallon drums were modified and utilized as enclosures within the pond. The bottoms of the drums were removed and the open-ended drum (termed “enclosure”) was placed into the pond and secured into pond sediments (~5-10 cm into sediments), capturing algae within. Algae were then added or removed from individual enclosures based on visual observation to achieve similar cell densities among the enclosures. Microcystis was the dominant alga in the pond (representing >99% of the algal assemblage) with Euglena sp. and Anabaena sp. present (representing <1% of the algal assemblage). Initial cell densities within enclosures ranged from 3.4-4.1x10⁶ cells/ mL (Table 3.1).
Copper Exposures

Laboratory Exposures

Cultured and field collected *Microcystis* were exposed to a series of concentrations of a chelated copper algaecide, Cutrine-Plus® Algaecide (Applied Biochemists [Arch Chemicals a Lonza Business], Alpharetta, GA) chelated with ethanolamine (Table 3.2). 1,000 mg Cu/L stock solutions were prepared from Cutrine-Plus® using Nanopure® water before initiation of toxicity experiments. 200 mL of cultured *M. aeruginosa* in BG-11 media and 200 mL of field collected *Microcystis* in site water were added to separate 250 mL acid washed borosilicate beakers. Appropriate volumes of stock copper solutions were added to each beaker to achieve targeted copper concentrations. Three replicate beakers were included for each copper exposure concentration. Cultured *M. aeruginosa* was exposed to seven concentrations: 0.02, 0.05, 0.07, 0.1, 0.2, 0.5 and 1.0 mg Cu/L as Cutrine-Plus®. Field collected *Microcystis* in site water was exposed to five concentrations, 0.1, 0.3, 0.5, 0.7, and 1.0 mg Cu/L as Cutrine-Plus®. Untreated controls did not receive copper additions. Acid soluble copper concentrations were measured at the initiation of the experiments to confirm targeted (nominal) exposures. Copper exposures for cultured *M. aeruginosa* were confirmed using an Agilent PSD 120 Flame Atomic Absorption Spectrometer (FAAS), and copper exposures for field collected algae were confirmed using a Perkin Elmer Optima 3100RL inductively coupled plasma-optical emission spectrometer (ICP-OES) with autosampler.
(APHA 2012). Percent error between measured and nominal copper exposures for laboratory experiments were 2-20% (Table 3.3).

Field Copper Exposures

Field copper exposures were prepared by estimating the volume of water within enclosures using a cylindrical segment equation (Equation 1). Enclosures (cylinders) were inserted into sloped sediments (44.3±4.6° slope) of the pond littoral zone. Height from the sediment surface to the water surface was measured at the top (h₁) and bottom (h₂) of the sloped sediments within each enclosure. The average volume (V) calculated for the 12 enclosures was 53.2±2.6 liters.

Equation 1. \[ V = \frac{1}{2} \pi r^2 (h_1 + h_2) \]

Cutrine-Plus® was then gravimetrically measured for each calculated enclosure volume to target exposure concentrations of 0.3, 0.5, and 0.8 mg Cu/L as Cutrine-Plus® with 3 replicate enclosures per concentration (n=3, N=12). The copper-algaecide was then added to each enclosure and mixed thoroughly. Acid soluble copper concentrations were measured at the initiation of the experiment to confirm exposures using an ICP-OES with autosampler (as previously described). Percent error between nominal and measured copper concentrations ranged from 1-21% (Table 3.3).

Algal Sampling

Responses of Microcystis to copper exposures were measured 1 through 5 days after treatment (1-5 DAT) in laboratory experiments with cultured M. aeruginosa and 1 and 4 DAT in laboratory experiments with field collected Microcystis and field water.
Samples were collected from beakers for measurement of MC-LR concentrations, chlorophyll $a$ concentrations, and cell densities. Cultured $M. aeruginosa$ samples were not mixed prior to collection as $Microcystis$ remained evenly distributed throughout the beaker due to buoyancy regulation with gas vacuoles. Although field collected $Microcystis$ were also gas vacuolated, the algae formed a thin layer at the surface of the beaker, requiring gentle mixing for collection of representative samples. Samples from field enclosures were collected prior to treatment and 1-6 DAT. Water in enclosures was mixed prior to sampling to evenly distribute $Microcystis$. Three 50 mL samples were collected from each enclosure and 25 mL was used for MC-LR$_{Tot}$ analysis, 30-50 mL was filtered for MC-LR$_{Aq}$ analysis (final volume of filtrate was recorded), and 5 mL was used for chlorophyll $a$ analysis.

**Algal Response Measures**

**MC-LR Quantification**

MC-LR concentrations were measured using high performance liquid chromatography (HPLC) with ultraviolet detection. The following HPLC parameters were selected from Sangolkar et al. (2006): A Dionex Ultimate 3000® HPLC was utilized with an Acclaim Polar Advantage II® column (5µm, 120Å, 4.6x250nm); parameters for analysis were: detection = 243 nm, temperature = 30°C, pressure = 1,500 psi, flow = 1 mL/min, and injection volume = 60 µL; solvents included Nanopure® H$_2$O/ 0.1% trifluoroacetic acid and HPLC grade acetonitrile. Peak area was measured at a detention time of 14.0-15.0 minutes and MC-LR concentrations were calculated by comparing
measured area units to known MC-LR standards (Beagle Bioproducts®, Columbus, Ohio). Known 100 µg /L MC-LR standards were measured between every 20 samples and variability was calculated to confirm measurement accuracy and precision. Method specific detection limits for MC-LR (lowest concentration at which the measured MC-LR concentration was significantly different from the solvent blank) (APHA 2012), using the 10-fold concentration factor in laboratory experiments was 1.0 µg MC-LR/L (ANOVA, α=0.05, p=0.0022). Method detection limits for field experiments were dependent on the final volume of filtered sample (30-40-fold concentration factor).

**Percentage $MC\text{-LR}_{Aq}$ of $MC\text{-LR}_{Tot}$**

Initial $MC\text{-LR}_{Aq}$ and $MC\text{-LR}_{Tot}$ concentrations were different among laboratory and field studies (Table 1). Therefore, the percentage $MC\text{-LR}_{Aq}$ of $MC\text{-LR}_{Tot}$ was calculated (Equation 2) to standardize comparisons between laboratory and field studies.

$$\text{Equation 2. Percentage } MC\text{-LR}_{Aq} = \left(\frac{MC\text{-LR}_{Aq}}{MC\text{-LR}_{Tot}}\right) \times 100$$

Where $MC\text{-LR}_{Aq}$ represents the measured aqueous MC-LR concentration and $MC\text{-LR}_{Tot}$ indicates the measured total MC-LR concentration.

**MC-LR Extraction and Concentration**

Aqueous MC-LR was separated by filtering 10 mL of laboratory sample from each beaker and approximately 25-50 mL of field collected sample from each enclosure through a Millipore® 0.45µm nitrocellulose filter. Total volumes of filtered field samples were recorded for calculation of $MC\text{-LR}_{Aq}$ concentrations. Filtrate was then concentrated by evaporation to dryness (using a water bath and compressed air) for approximately 8-
12 hours. The concentrate was then re-suspended in 1 mL of 80:20 Nanopure® water and HPLC grade acetonitrile, vortexed to mix, and transferred to 2 mL HPLC vials for HPLC quantification.

For laboratory cultured *M. aeruginosa*, cellular MC-LR (MC-LR<sub>Cell</sub>) was extracted by pipetting 10 mL of sample from each beaker and centrifuging at 3,300 rpm for 10 minutes. Supernatant was discarded and MC-LR was extracted from the algal pellet. MC-LR<sub>Tot</sub> was determined by adding measured MC-LR<sub>Cell</sub> to measured MC-LR<sub>Aq</sub>: MC-LR<sub>Cell</sub> + MC-LR<sub>Aq</sub> = MC-LR<sub>Tot</sub>. For field collected *Microcystis*, algal cells could not be separated by centrifuging. Therefore, collected whole samples were evaporated and MC-LR<sub>Tot</sub> was extracted from dried material. For field collected algae, MC-LR<sub>Cell</sub> = MC-LR<sub>Tot</sub> − MC-LR<sub>Aq</sub>.

For MC-LR extraction, algal pellets and dried algal material were frozen at -80°C, thawed, evaporated, and re-suspended in 1 mL of 80:20 Nanopure® water and HPLC grade acetonitrile. Re-suspended pellet was stored at 4°C overnight (approximately 12 hours) in solvent and then sonicated using a Branson® 5210 sonic bath for 5 minutes. Samples were then centrifuged and filtered through a Millipore® 0.45µm nitrocellulose filter into 2 mL HPLC vials for MC-LR quantification.

*Cell Viability: Chlorophyll a and Cell Density*

5 mL subsamples of *Microcystis* were collected from exposures in both laboratory and SFSs for analysis of algal viability. Chlorophyll *a* was measured using a spectrophotometric method (APHA 2012) on a SpectraMax M2 spectrofluorometer
Chlorophyll a standards were acquired from Sigma Aldrich (C6144). Cell density was measured using light microscopy with an improved Neubauer hemocytometer (Hausser Scientific Co. Horsham, PA 19044). Field collected colonial *Microcystis* was sonicated (Fisher Scientific, Sonic Dismembrator 550) for approximately five seconds prior to counting in order to separate colonial cells for more accurate enumeration.

**Percent Change: Cell Density and Chlorophyll a**

Changes in cell densities and chlorophyll a concentrations following copper exposures were calculated as a percent change from initial pre-treatment values (Equation 3).

\[
\text{Equation 3. } \%\Delta = \left(\frac{C - C_0}{C_0}\right) \times 100
\]

Where \(C\) indicates the measured cell density or chlorophyll a concentrations at 4 DAT with Cutrine-Plus®, \(C_0\) is the initial pre-treatment cell density or chlorophyll a concentration, and \(\%\Delta\) indicates the percent change in cell density or chlorophyll a concentration from the initial, pre-treatment value. A negative value indicates a decrease in cell density or chlorophyll a, while a positive value indicates an increase.

**Statistical Analysis**

Slopes and EC\(_{50}\)s were calculated using measured copper concentrations for *Microcystis* responses in terms of MC-LR\(_{Aq}\) 1 DAT. MC-LR\(_{Aq}\) concentrations in relation to copper exposure concentration were analyzed using non-linear regression with a sigmoidal, 4P logistic fit function (JMP v.11). Inflection points calculated were
synonymous with EC$_{50}$ values. Differences in extent of MC-LR release, chlorophyll $a$
concentrations, and cell densities between copper exposures within each study (i.e.
laboratory and field) were calculated using ANOVA and Tukey’s pairwise comparison
(JMP v.11). Measured equal variance and normality of distribution were analyzed using
Levene’s test and the Shapiro-Wilk test, respectively.
Results

Responses of Microcystis

MC-LRAq and Copper Relationships

MC-LR release occurred within 24h in laboratory experiments and SFSs following exposures of copper-based algaecides. MC-LR release (i.e. MC-LRAq concentration) was positively correlated with copper concentration in all experiments (i.e. MC-LRAq concentrations increased as copper concentrations increased; Table 3.4, Figure 3.1). The 24h median effects concentration (24h-EC50) where 50% of MC-LRtot was in the aqueous phase (MC-LRAq) was lowest in laboratory experiments with cultured M. aeruginosa and formulated BG-11 media (0.09 mg Cu/L). The 24h-EC50 for laboratory experiments with field collected Microcystis, 0.30 (95% CI = 0.18-0.42) mg Cu/L, was not different from the 24h-EC50 in SFSs with the same Microcystis, 0.38 (95% CI = 0.27-0.48) mg Cu/L, based on confidence intervals (Table 3.4). Potency slopes representing changes in MC-LRAq concentrations with changes in copper concentrations were also greatest (steepest slopes) in laboratory experiments with cultured M. aeruginosa (Table 3.4). Slopes for cultured M. aeruginosa, field collected Microcystis in the laboratory, and Microcystis in SFSs were 77.3, 8.0, and 13.3 respectively (Table 3.4).

Following confirmation of targeted copper concentrations in each experimental enclosure, it was determined that the targeted copper concentration of 0.3 mg Cu/L was not achieved in a single enclosure (i.e. one of three replicates in the 0.3 mg Cu/L treatment) (measured = 0.22 mg Cu/L). Microcystis responses (cell density and
chlorophyll $a$ concentrations) for the enclosure violated Levene’s test for equal variances ($p=0.002, \alpha=0.05$) and the Shapiro-Wilk test for normal distribution ($p=0.0047, \alpha=0.05$, JMP v.11). Removal of the enclosure resulted in normal distribution (Shapiro-Wilk; $p=0.827, \alpha=0.05$) and equal variances (Levene; $p=0.506, \alpha=0.05$). Based on failure to achieve the targeted concentration and statistical confirmation of algal responses measured from the enclosure as outliers, the data for the single enclosure were not included in the analysis.

**MC-LRAq Following Copper Exposures**

Initial MC-LRAq and MC-LRTot concentrations were different among laboratory and field studies (Table 3.1). Therefore, results are discussed as the percentage MC-LRAq of MC-LRTot to standardize comparisons of MC-LRAq release following copper exposures (Table 3.6). Measured MC-LRAq concentrations are presented in Table 3.5. The percentage of MC-LRAq increased with copper concentrations within the range of effective copper concentrations (i.e. where chlorophyll $a$ concentrations and cell densities reached a maximum response and no longer decreased with increased copper; Tables 3.5 and 3.6, Figures 3.1 and 3.2). The lowest effective copper concentrations for laboratory cultured *M. aeruginosa*, field collected *Microcystis* in the laboratory, and *Microcystis* in SFSs were 0.07, 0.3, and 0.3 mg Cu/L respectively. Corresponding percentages of MC-LRAq were 15±19, 59±13, and 29±18% of MC-LRTot respectively (Table 3.5, Figure 1). Maximum release of MC-LR to the aqueous phase occurred at copper concentrations greater than 0.2, 0.3, and 0.3 mg Cu/L for cultured *M. aeruginosa* in laboratory...
experiments, field collected *Microcystis* in laboratory experiments, and *Microcystis* in field experiments, respectively (Tables 3.5 and 3.6, Figure 3.1). Percentages of MC-LR\(_{Aq}\) at the highest targeted copper exposures for cultured *M. aeruginosa* in laboratory experiments (1.0 mg Cu/L), field collected *Microcystis* in laboratory experiments (1.0 mg Cu/L), and *Microcystis* in SFSs (0.8 mg Cu/L) were approximately 77±3, 84±3, and 77±10%, respectively (Table 3.6, Figure 3.1).

MC-LR\(_{Aq}\) concentrations increased significantly following algaecide exposures compared to the control in all studies (p≤0.0001, α=0.05; Figure 3.1, Table 3.5-3.6). However, MC-LR\(_{Aq}\) concentrations were dependent on copper concentration and were significantly less following exposures to lowest effective copper concentrations (0.07, 0.3, and 0.3 mg Cu/L) compared to highest copper exposures (1.0, 1.0, and 0.8 mg Cu/L) (p=0.0063-0.041, α=0.05; Figures 3.1-3.2, Tables 3.5-3.6).

**Chlorophyll a and cell density**

Control of *Microcystis* (i.e. chlorophyll a concentrations and cell densities no longer decreased with increased copper concentration; Table 3.5-3.6, Figure 3.1-3.2) was achieved at 0.07, 0.3, and 0.3 mg Cu/L for laboratory cultured *M. aeruginosa*, field collected *Microcystis* in the laboratory, and *Microcystis* in SFSs, respectively (Figure 3.2, Table 3.5-3.6). Cell densities and chlorophyll a concentrations were significantly less than untreated controls 4 DAT at all effective copper exposures in both laboratory and SFSs (p=<0.0001-0.048, α=0.05). In laboratory exposures with cultured *M. aeruginosa* cell density and chlorophyll a decreased approximately 16-34% (3.2-4.2x10^5 cells/mL).
and 65-71% (150-180 µg chlorophyll a /L) 4 DAT from pretreatment concentrations (4.5x10^6 cells/mL and 510 µg chlorophyll a /L). Laboratory exposures of field-collected algae to copper resulted in a 92-100% decrease in cell density (BDL [detection: 2.0 x10^3] to 8.3x10^5 cells/mL) and a 52-75% decrease in chlorophyll a (65-125 µg chlorophyll a /L) from pretreatment concentrations (1.1x10^6 cells/mL and 258 µg chlorophyll a /L).

*Microcystis* exposure to copper algaecides in SFSs resulted in decreased chlorophyll a concentrations and cell densities by 83-88% (4.3-6.3x10^5 cells/mL) and 26-59% (129-135 µg chlorophyll a /L) 4 DAT, respectively, from pre-treatment values (~3.7 x10^6 cells/mL and 320 µg chlorophyll a /L).
Discussion

Field derived *Microcystis* responded (in terms of both MC-LR release and cell viability) to 96-h copper-based algaecide exposures at approximately the same concentrations in both preliminary laboratory experiments and SFSs. Maximum response of *Microcystis* (as indicated by chlorophyll *a* and cell density) was achieved at 0.3 mg Cu/L and the 24h-EC_{50} for MC-LR release was 0.3 and 0.38 mg Cu/L for laboratory and field experiments, respectively. Potency slopes were also similar following exposures of field *Microcystis* to copper in laboratory experiments and SFS, 8.0 (-1.0, 17.0) and 13.3 (1.3, 17.8), respectively. By utilizing the same *Microcystis* and water in both laboratory and field experiments, differences in potential exposure modifying factors (i.e. water characteristics [alkalinity and hardness, USEPA 2007] and specific algal sensitivity) are minimized. Therefore, comparable copper exposures to the algae were achieved in both laboratory and field experiments, resulting in comparable responses. Analogous responses of field collected *Microcystis* to similar copper exposures in the laboratory and field supports previous studies which indicate that preliminary laboratory experiments of field-collected algae can inform effective concentrations in field applications for specific algae within site parameters (i.e. water characteristics and algal sensitivities; Mastin et al. 2002, Murray-Gulde et al. 2002, Bishop and Rodgers 2012).

In contrast, laboratory cultured *M. aeruginosa* in formulated BG-11 medium was more sensitive to 96-h copper exposures than field derived *Microcystis*, as indicated by viability measures (chlorophyll *a* and cell density) and MC-LR release (24h-EC_{50}s and
potency slopes). Differences between sensitivities of field collected *Microcystis* in site water and cultured *M. aeruginosa* in BG-11 medium may have been due to differing characteristics among field waters and formulated medium. Water characteristics, primarily alkalinity and hardness (which were higher in field water than in BG-11 medium) influence the exposure and subsequent toxicity of copper to aquatic organisms (USEPA 2007). Sensitivities of different strains of the same algal species may also play a role in differing responses of laboratory cultured *M. aeruginosa* compared to field collected. Field-collected *Microcystis* in this study was colonial and surrounded by mucilage compared to the single-celled strain cultured in the laboratory. Extracellular, polysaccharide mucilage produced by cyanobacteria is hypothesized to aid in excluding and regulating foreign substances, including metals, from entering cells (Reynolds 2007).

Despite differences in sensitivities between laboratory cultured and field collected *Microcystis*, positive sigmoidal relationships between MC-LR release (MC-LRAq) and copper concentration were measured in all experiments (laboratory and field). This sigmoidal exposure-response relationship to copper also describes concentration dependent algal responses such as chlorophyll a concentration, cell density, and cell membrane integrity following copper exposure (Calomeni and Rodgers 2014). At all copper concentrations that effectively decreased cell densities and chlorophyll a concentrations in *M. aeruginosa* (0.07-1.0 mg Cu/L for laboratory cultured *Microcystis* and 0.3-1.0 mg Cu/L for field collected *Microcystis* in laboratory and SFSs), a portion of MC-LRtot was released to the aqueous phase. However, the extent of release was related
to copper concentrations where MC-LR release at the lowest effective copper concentrations (0.07-0.3 mg Cu/L) was significantly less than MC-LR release at the highest copper exposures (0.8-1.0 mg Cu/L). The positive relationship between copper concentration and MC-LR\textsubscript{Aq} at effective algaecide concentrations demonstrates that lower copper concentrations were as effective as higher concentrations in controlling \textit{Microcystis} while minimizing the amount of MC-LR released to aqueous phase.

Fundamentally, the incremental release of MC-LR from \textit{Microcystis} within a range of copper concentrations where cell density and chlorophyll \textit{a} responses remained constant may be due to multiple mechanisms by which copper elicits toxicity to cells. Copper elicits toxicity through inhibition of cell functions by binding to non-copper specific proteins (Stevenson et al. 2013) and disruption of photosynthetic processes (Qian et al. 2010, Jancula and Marsalek 2011). Copper can cause loss of cell membrane integrity (Fan et al. 2014, Zhou et al. 2013) through osmotic imbalance and production of reactive oxygen species (ROS; Qian et al. 2010), which likely results in MC-LR release. At relatively high copper concentrations (0.5-1.0 mg Cu/L), visible bleaching of cells occurred within hours of copper treatment and blue pigmentation (likely phycocyanin) could be seen in surrounding waters. Phycocyanin is a water soluble photosynthetic pigment of cyanobacteria that can be released, similarly to MC, from cells to surrounding water following cell lysis (Barrington et al. 2013). Lower effective copper concentrations (0.07-0.3 mg Cu/L) did not result in rapid (<24h) chlorosis of cells or pigmentation of surrounding water, but within 4 days had decreased algal viability to the same extent.
Based on measured responses of Microcystis and visual observations in this study, we hypothesize that copper mechanisms of action shift with increased copper exposures where lower exposures may result in disruption of photosynthesis and other basal cellular functions before cell lysis and loss of membrane integrity occurs.

Multiple lines of evidence gathered from both laboratory and SFSs utilizing two different strains of Microcystis support the hypothesis that MC-LR release following copper exposure is concentration dependent. Although specific concentrations required to control Microcystis did not directly translate between laboratory and field experiments that utilized different algae and water; analyses of Microcystis in laboratory and SFSs that used the same algae and site water resulted in similar responses. This emphasizes that preliminary laboratory experiments with site-specific algae and water/ medium are useful to predict effective exposure concentrations for field treatments, both in terms of algal viability and concentrations that will minimize release of MC-LR. As the concentration required to control the targeted algae is site specific and influenced by both water and algal characteristics, this further emphasizes the importance of preliminary experiments for targeted, site specific copper applications.
Conclusions

MC-LR release (MC-LR$_{Aq}$) was positively correlated with copper concentrations in laboratory experiments with cultured *M. aeruginosa* in formulated BG-11 medium, laboratory experiments with field collected *Microcystis* and water, and in *in situ* SFSs with *Microcystis*. The incremental release of MC-LR from *Microcystis* within a range of effective copper concentrations at which cell density and chlorophyll a responses remained constant demonstrates that lower copper concentrations were as effective as higher concentrations in controlling *Microcystis* while decreasing the amount of MC-LR released to aqueous phase. Although laboratory cultures of *M. aeruginosa* in BG-11 medium were more sensitive to copper exposures than field algae in site water, laboratory experiments with field collected algae produced similar results to SFSs with the same algae and water. This emphasizes the utility of preliminary laboratory experiments for predicting *Microcystis* responses in the field. The results also highlight that using copper more efficiently (i.e. utilizing the minimum concentration needed for control of the target algae) decreased not only the amount of copper-algaecide required, but can also minimize MC-LR release. Results from this experiment and the approach are useful for resource managers requiring rapid relief from noxious algal issues and management of risks associated with both MC-LR$_{Aq}$ and MC-LR$_{Tot}$.
References


Microcystis in field enclosures formed a thin layer (<1.0 cm) on the water surface. Within the algal layer during daylight hours, dissolved oxygen concentrations as high as 9.6 mg O$_2$/L were measured. Immediately below the algal layer, dissolved oxygen concentrations were less than 1 mg O$_2$/L.

Water temperatures in the field ranged from ~19ºC prior to sunrise (~7:00am) to as high as ~27ºC at mid-day (~1:00pm).

Light intensity was measured at the water surface and the range represents the highest and lowest light intensity measured across all beakers or enclosures. Light in the laboratory was provided by cool-white fluorescent bulbs (Residential Ecolux 40 W, GE).

Light:dark duration in the field was based on approximate day length in Anderson, South Carolina from September 18-25, 2015.
Table 3.2 Characteristics of copper sulfate pentahydrate (CuSO₄) and Cutrine-Plus®

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CuSO₄•5H₂O</th>
<th>Cutrine®-Plus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active ingredient</td>
<td>Copper</td>
<td>Copper</td>
</tr>
<tr>
<td>% Active ingredient</td>
<td>23.7</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximum label concentration as copper</td>
<td>2 mg/L&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 mg/L&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formulation</td>
<td>Copper sulfate pentahydrate</td>
<td>Copper ethanolamine complex&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Appearance</td>
<td>Blue crystals</td>
<td>Blue liquid</td>
</tr>
<tr>
<td>Water solubility</td>
<td>415,997 mg/L</td>
<td>Miscible&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Boiling point (°C)</td>
<td>106&lt;sup&gt;**&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific gravity (g/cm³)</td>
<td>1.21&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.22</td>
</tr>
<tr>
<td>pH</td>
<td>3.22&lt;sup&gt;**&lt;/sup&gt;</td>
<td>10.3-10.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Physical and chemical characteristics are of the original compound unless otherwise noted.

<sup>a</sup> Cutrine®-Plus can mix with water in all proportions.
<sup>**</sup> Physical and chemical characteristics of saturated solution
<sup>a</sup> Applied Biochemists product label
<sup>b</sup> Applied Biochemists MSDS
<sup>c</sup> Murray-Gulde et al. 2002
<sup>c</sup> Chem One Ltd. product label
Table 3.3 Measured and Nominal (Targeted) Copper Concentrations from Cutrine-Plus® Exposures

<table>
<thead>
<tr>
<th>Nominal (mg Cu/L)</th>
<th>0</th>
<th>0.05</th>
<th>0.07</th>
<th>0.1</th>
<th>0.2</th>
<th>0.5</th>
<th>1.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured 1 (mg Cu/L)</td>
<td>0.02</td>
<td>0.053</td>
<td>0.079</td>
<td>0.127</td>
<td>0.213</td>
<td>0.473</td>
<td>0.873</td>
</tr>
</tbody>
</table>

Table 3.4 24h-EC50s, Slopes, and R² for Microcystis (MC-LRₐq) exposed to copper as Cutrine-Plus®

<table>
<thead>
<tr>
<th></th>
<th>Laboratory</th>
<th>Field Algae in</th>
<th>Field Enclosures</th>
</tr>
</thead>
<tbody>
<tr>
<td>24h-EC₅₀</td>
<td>0.09 (0.08, 0.10)</td>
<td>0.30 (0.18, 0.42)</td>
<td>0.38 (0.27, 0.48)</td>
</tr>
<tr>
<td>Slope (95%)</td>
<td>77.3 (34.2, 115.6)</td>
<td>8.0 (-1.0, 17.0)</td>
<td>13.3 (1.3, 17.8)</td>
</tr>
<tr>
<td>R²</td>
<td>0.92</td>
<td>0.91</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Laboratory copper exposures were distributed from one initial stock exposure among three replicate beakers; therefore, only one exposure per copper concentration was measured to confirm target exposure was reached (A-B). Field copper exposures were applied to individual enclosures of different volumes; therefore, copper exposures in each individual replicate were measured and confirmed (C).

Aqueous microcystin-LR (MC-LRₐq), median effects concentration where 50% of MC-LRₐq is MC-LRₐq(EC₅₀). EC₅₀ represent mg Cu/L as Cutrine-Plus®
### Table 3.5 Measured *Microcystis* responses (MC-LR\textsubscript{aq} concentrations, chlorophyll \(a\) concentrations, and cell densities) following copper exposure

<table>
<thead>
<tr>
<th></th>
<th>0 µg Cu/L</th>
<th>0.05 µg Cu/L</th>
<th>0.07 µg Cu/L</th>
<th>0.1 µg Cu/L</th>
<th>0.2 µg Cu/L</th>
<th>0.3 µg Cu/L</th>
<th>0.5 µg Cu/L</th>
<th>0.7 µg Cu/L</th>
<th>0.8 µg Cu/L</th>
<th>1.0 µg Cu/L</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MC-LR\textsubscript{aq}</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1±0</td>
<td>4±1</td>
<td>8±10</td>
<td>28±3</td>
<td>33±6</td>
<td>·</td>
<td>40±3</td>
<td>·</td>
<td>·</td>
<td>40±2</td>
</tr>
<tr>
<td><strong>MC-LR\textsubscript{aq}</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2±1</td>
<td>·</td>
<td>·</td>
<td>3±1</td>
<td>5±1</td>
<td>9±1</td>
<td>10±1</td>
<td>·</td>
<td>8±1</td>
<td></td>
</tr>
<tr>
<td><strong>MC-LR\textsubscript{aq}</strong>&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.1±0.1</td>
<td>·</td>
<td>·</td>
<td>·</td>
<td>1.8±1.1</td>
<td>4.2±0.9</td>
<td>·</td>
<td>4.7±0.5</td>
<td>·</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll (a)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1153±136</td>
<td>514±173</td>
<td>150±13</td>
<td>115±1</td>
<td>177±62</td>
<td>·</td>
<td>180±8</td>
<td>·</td>
<td>173±25</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll (a)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>258±15</td>
<td>·</td>
<td>·</td>
<td>196±3</td>
<td>·</td>
<td>125±21</td>
<td>65±17</td>
<td>77±9</td>
<td>·</td>
<td>71±12</td>
</tr>
<tr>
<td>Chlorophyll (a)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>320±81</td>
<td>·</td>
<td>·</td>
<td>·</td>
<td>135±45</td>
<td>132±47</td>
<td>·</td>
<td>129±64</td>
<td>·</td>
<td></td>
</tr>
<tr>
<td>Cell Density&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.4±0.1x10(^7)</td>
<td>5.5±2.2x10(^5)</td>
<td>3.4±0.6x10(^5)</td>
<td>3.8±0.3x10(^5)</td>
<td>4.2±0.9x10(^5)</td>
<td>·</td>
<td>3.2±0.2x10(^5)</td>
<td>·</td>
<td>3.9±0.2x10(^5)</td>
<td></td>
</tr>
<tr>
<td>Cell Density&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.1±0.2x10(^6)</td>
<td>·</td>
<td>·</td>
<td>1.1±0.1x10(^6)</td>
<td>·</td>
<td>8.3±0.3x10(^5)</td>
<td>BDL</td>
<td>BDL</td>
<td>·</td>
<td>BDL</td>
</tr>
<tr>
<td>Cell Density&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.4±0.3x10(^6)</td>
<td>·</td>
<td>·</td>
<td>·</td>
<td>6.3±1.4x10(^5)</td>
<td>5.1±1.2x10(^5)</td>
<td>·</td>
<td>4.3±1.6x10(^5)</td>
<td>·</td>
<td></td>
</tr>
</tbody>
</table>

(-) indicates exposures were not conducted at specified concentration. MC-LR\textsubscript{aq} = (µg/L, 1DAT), chlorophyll \(a\) = (µg/L, 4DAT), cell density = (cells/mL, 4DAT)

<sup>a</sup> Responses of cultured *Microcystis* in formulated BG-11 medium following laboratory copper exposures

<sup>b</sup> Responses of field collected *Microcystis* in site water following laboratory copper exposures

<sup>c</sup> Responses of *Microcystis* in simulated field studies (enclosures) following copper exposures
Table 3.6 Percent MC-LR\textsubscript{Aq} of MC-LR\textsubscript{Tot} 1DAT and percent change 4DAT in cell density and chlorophyll \(a\) from pre-treatment values

<table>
<thead>
<tr>
<th></th>
<th>0 (\mu g) Cu/L</th>
<th>0.05 (\mu g) Cu/L</th>
<th>0.07 (\mu g) Cu/L</th>
<th>0.1 (\mu g) Cu/L</th>
<th>0.2 (\mu g) Cu/L</th>
<th>0.3 (\mu g) Cu/L</th>
<th>0.5 (\mu g) Cu/L</th>
<th>0.7 (\mu g) Cu/L</th>
<th>0.8 (\mu g) Cu/L</th>
<th>1.0 (\mu g) Cu/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC-LR\textsuperscript{a}</td>
<td>2±0</td>
<td>8±2</td>
<td>15±19</td>
<td>53±7</td>
<td>63±11</td>
<td>·</td>
<td>78±5</td>
<td>·</td>
<td>·</td>
<td>77±3</td>
</tr>
<tr>
<td>MC-LR\textsuperscript{b}</td>
<td>22±9</td>
<td>·</td>
<td>32±8</td>
<td>·</td>
<td>59±13</td>
<td>80±4</td>
<td>85±1</td>
<td>·</td>
<td>84±3</td>
<td></td>
</tr>
<tr>
<td>MC-LR\textsuperscript{c}</td>
<td>3±2</td>
<td>·</td>
<td>·</td>
<td>·</td>
<td>29±18</td>
<td>62±12</td>
<td>·</td>
<td>77±10</td>
<td>·</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll (a)\textsuperscript{a}</td>
<td>124±27</td>
<td>0±34</td>
<td>-71±3</td>
<td>-78±0</td>
<td>-66±12</td>
<td>·</td>
<td>-65±2</td>
<td>·</td>
<td>·</td>
<td>-66±5</td>
</tr>
<tr>
<td>Chlorophyll (a)\textsuperscript{b}</td>
<td>0±6</td>
<td>·</td>
<td>-24±1</td>
<td>·</td>
<td>-52±8</td>
<td>-75±6</td>
<td>-70±4</td>
<td>·</td>
<td>-72±5</td>
<td></td>
</tr>
<tr>
<td>Chlorophyll (a)\textsuperscript{c}</td>
<td>0±25</td>
<td>·</td>
<td>·</td>
<td>·</td>
<td>-26±55</td>
<td>-59±15</td>
<td>·</td>
<td>-59±20</td>
<td>·</td>
<td></td>
</tr>
<tr>
<td>Cell Density\textsuperscript{a}</td>
<td>178±6</td>
<td>10±4</td>
<td>-24±1</td>
<td>-25±6</td>
<td>-16±18</td>
<td>·</td>
<td>-34±4</td>
<td>·</td>
<td>·</td>
<td>-23±6</td>
</tr>
<tr>
<td>Cell Density\textsuperscript{b}</td>
<td>0±22</td>
<td>·</td>
<td>-2±12</td>
<td>·</td>
<td>-92±3</td>
<td>-100±0</td>
<td>-100±0</td>
<td>·</td>
<td>-100±0</td>
<td></td>
</tr>
<tr>
<td>Cell Density\textsuperscript{c}</td>
<td>-15±7</td>
<td>·</td>
<td>·</td>
<td>·</td>
<td>-83±4</td>
<td>-86±3</td>
<td>·</td>
<td>-88±5</td>
<td>·</td>
<td></td>
</tr>
</tbody>
</table>

(-) indicates exposures were not conducted at specified concentration. MC-LR\textsubscript{Aq} = (\(\mu g/L\), 1DAT), chlorophyll \(a\) = (\(\mu g/L\), 4DAT), cell density = (cells/mL, 4DAT), MC-LR values represent percentage MC-LR\textsubscript{Aq} of MC-LR\textsubscript{Tot} 1 DAT. Chlorophyll \(a\) and cell density values represent the percent change 4 DAT from pre-treatment values

\textsuperscript{a} Responses of cultured \textit{M. aeruginosa} in formulated BG-11 medium following laboratory copper exposures

\textsuperscript{b} Responses of field collected \textit{Microcystis} in site water following laboratory copper exposures

\textsuperscript{c} Responses of \textit{Microcystis} in \textit{in situ} simulated field studies (enclosures) following copper exposure
Figure 3.1 Proportion (%) of MC-LR\textsubscript{Cell} and MC-LR\textsubscript{Aq} of MC-LR\textsubscript{Tot} (represented by full bar) measured 1 day DAT (a, c, e) with copper as Cutrine Plus\textsuperscript{®}. Laboratory cultured \textit{M. aeruginosa} and formulated BG-11 media (a), field collected \textit{Microcystis} and water in laboratory conditions (b), and field collected algae in \textit{in situ} field enclosures. Error bars = 1 standard deviation, n=3.
Figure 3.2 Microcystis cell density (a, c, e) and chlorophyll a concentrations (b, d, f) measured 4 days after treatment (4 DAT) with Cutrine Plus®. Laboratory cultured *M. aeruginosa* and formulated BG-11 media (a, b), field collected *Microcystis* and water in laboratory conditions (c, d), and field collected algae in field mesocosms (e, f). Error bars = 1 standard deviation, n=3
CHAPTER 4

INFLUENCE OF COPPER ALGAECIDE CONCENTRATION AND FORMULATION ON MICROCYSTIN-LR DEGRADATION

Abstract

Impediment of bacterial degradation of microcystin-LR (MC-LR) in aquatic systems due to copper algaecide exposures has been hypothesized. This assumption can influence decisions to use copper based algaecides for management of MC-LR producing cyanobacteria and is based on relatively few studies. Potential influences of copper algaecide concentrations and formulations on MC-LR degradation and on bacterial community composition were investigated in this study. In laboratory experiments, the MC-LR producing cyanobacterium (*Microcystis aeruginosa*) was exposed to a series of copper concentrations (0, 0.1, 0.5, 1.0, and 5.0 mg Cu/L) using three copper-based algaecides, a copper salt (CuSO₄), chelated copper-ethanolamine (Cutrine-Plus®), and chelated copper-citrate and copper-gluconate (Algimycin-PWF®). Following copper exposures, aqueous and total MC-LR (MC-LRₜₒₜ) concentrations were measured for 7 days, and rates and extents of MC-LRₜₒₜ degradation were calculated. Following exposure to 0, 0.1, 0.5, and 1.0 mg Cu/L, MC-LRₜₒₜ concentrations decreased to method detection (2 µg/L MC-LR) from pre-treatment concentrations (~86-121 µg/L MC-LRₜₒₜ) within 96h. Following exposure to 5.0 mg Cu/L, MC-LRₜₒₜ was degraded slower compared to other treatments and decreased ~30% 7 d post-copper exposure. Diversity of
bacterial assemblages decreased following exposures of copper algaecides greater than 0.1 mg Cu/L as CuSO₄, Cutrine-Plus®, Algimycin-PWF®. Relative abundance of certain groups of MC-degrading bacteria identified in treatments increased with increasing copper concentration, suggesting they may be less sensitive to copper exposures than other, MC-LR and non MC-degrading heterotrophic bacteria present in the assemblage. Results of this study revealed that copper concentration can influence degradation rates of MC-LR, however this influence was not significantly different from untreated controls within copper algaecide concentrations currently registered for use (0.1 – 1.0 mg Cu/L) of the tested copper-algaecides (CuSO₄, Cutrine-Plus®, Algimycin-PWF®). Copper formulation did not significantly alter degradation rates or bacterial composition at comparable copper treatments. These data augment our understanding of the influences of copper algaecide exposures on MC-LR degradation, and can be used to inform more accurate risk evaluations and use of copper-based algaecides for management of MC-LR producing *M. aeruginosa*. 
Introduction

Microcystin (MC) is a cyanobacterial produced hepatotoxin that poses both ecological and human health risks in water resources (Carmichael 2001, WHO 2003). There are over 80 structural variants of MCs and microcystin-LR (MC-LR; Table 4.1) is among the most problematic and toxic of the MC congeners (WHO 2003). As an endotoxin, MC-LR exists in nature in two forms, as either cellular MC-LR (MC-LR_{Cell}) or aqueous MC-LR (MC-LR_{Aq}) outside of the cell. The sum of MC-LR_{Cell} and MC-LR_{Aq} is operationally defined as total MC-LR (MC-LR_{Tot}). Typically, MC-LR is contained within the cyanobacterial cell as MC-LR_{Cell} until cell death (Zohary and Pais Madeira 1990, Chorus and Bartram 2000) which may occur naturally (apoptosis, autolysis), following chemical treatment from algaecides, potable and wastewater treatment processes, and accidental or intentional ingestion and digestion of cells by organisms (Beasley 1989). Following release of MC-LR to the aqueous phase in aquatic systems, MC-LR_{Aq} is subject to transfers or transformations through bacterial degradation, dilution and dispersion, photolysis, and sorption (Table 4.1). However, the primary removal pathway for MC-LR_{Aq} in aquatic systems is bacterial degradation with half-lives ranging from approximately 1 to 14 days (Table 4.1).

Risks associated with MC-LR in aquatic systems can be mitigated by decreasing densities of MC-LR-producing cyanobacteria. Algaecides are often employed to rapidly restore water resource uses that have been compromised by noxious cyanobacteria growths. Copper is a widely used active ingredient in algaecides, bactericides and
fungicides, with its antimicrobial (algaecidal and bactericidal) properties recognized as early as 1904 (Clark and Gage, 1905). Copper can elicit toxicity in bacteria/cyanobacteria through multiple mechanisms of action including inhibition of protein structure, function and activity, increased membrane permeability, membrane lipid peroxidation, and production of reactive oxygen species (Stevenson et al. 2013). The antimicrobial properties of copper responsible for its effectiveness in decreasing cyanobacteria densities, have also led to concerns regarding potential influences of copper from algaecide exposures on bacterial degradation of MC-LR. It is hypothesized that copper algaecides utilized to decrease growths of noxious cyanobacteria may also decrease densities of resident heterotrophic bacteria (including those capable of MC-LR degradation) which may result in MC-LR persistence due to a lag time prior to degradation and/or decreased degradation rates (Jones and Orr, 1994).

This assumption of MC-LR persistence following copper algaecide applications is based primarily on a single field study where a copper algaecide treatment of an unspecified concentration resulted in persistence of MC-LR in a drinking water reservoir for approximately 21 days (Jones and Orr, 1994). Due to increased awareness of MC-LR related risks and regulatory intervention in MC-LR affected waters, data are needed to understand the relative influence of copper algaecide applications on MC-LR degradation. More definitive investigation of the influence of copper on MC-LR degradation following copper algaecide exposures will allow for more informed decisions for managing MC-LR-producing cyanobacteria with copper-based algaecides.
Characteristics of copper exposures that have thus far been overlooked hypothesized to influence degradation rates of MC-LR following algaecide applications include copper concentrations and formulations. Organism responses (i.e. cyanobacteria and bacteria) to copper are concentration dependent and relationships between copper concentrations and organism responses have been established across a range of taxonomic groups (vertebrates, invertebrates, eukaryotic algae, cyanobacteria, and bacteria) (EPA 2007, Geer et al. 2016, Calomeni and Rodgers 2014). Therefore, it is reasonable to hypothesize that a relationship exists between bacterial density/activity, copper concentration, and rates of bacterial mediated degradation of MC-LR.

Copper algaecide formulation may also influence MC-LR degradation. Multiple forms of copper algaecides are registered for use including copper salts (CuSO₄) and chelated copper compounds such as copper-ethanolamine (Cutrine-Plus®) and copper-citrate and gluconate (Algimycin-PWF®) (Table 4.2). Algae may respond differently depending on the copper algaecide formulation to which they are exposed; for algae, chelated copper compounds are often more potent than CuSO₄ (Murray-Gulde et al. 2002, Calomeni et al. 2014). Differences in sensitivities between copper formulations have been measured in other taxonomic groups as well (Murray-Gulde et al. 2002, Calomeni et al. 2014). Based on responses of other taxonomic groups to a variety of copper formulations, heterotrophic bacteria may also respond differently depending on the copper form to which they are exposed. Metals may be more readily transported across cell membranes when complexed with ligands (Campbell et al. 2002). Chelated
copper compounds remain in solution for longer durations as compared to un-complexed cupric ions (Cu$^{2+}$), which can increase potency of copper algaecides (Masuda and Boyd 1993, Murray-Gulde et al. 2002). Factors influencing algaecide exposures (i.e. formulation and concentration) may alter responses of microorganisms to copper and ultimately could be manifested as differences in MC-LR degradation. Un-confounded experiments are needed to test the influence of these specific factors on MC-LR degradation.

Laboratory toxicity experiments are frequently used in measuring exposures and responses of both target and non-target organisms to pesticides (Rand et al. 1995, Calomeni et al. 2014, Geer et al. 2016). In the present study, laboratory experiments facilitated testing influences of a wide range of copper formulations and concentrations on MC-LR degradation while maintaining adequate replication and control of potentially confounding factors such as photolysis and sorption of MC-LR (Tsuji et al. 1994 and 1995, Wu et al. 2011, Miller et al. 2001, Morris et al. 2000).

Effects of copper algaecide concentration and formulation on MC-LR degradation can be determined analytically by measuring changes in MC-LR concentrations over time. In addition, bacterial analyses provide valuable information to discern changes in bacterial community composition (Haakensen et al. 2015, Kinely et al. 2016) following chemical exposures (i.e. copper). The premise that copper algaecide applications result in persistence of MC-LR due to effects on heterotrophic MC-degrading bacteria (i.e. strain(s) within Arthrobacter, Bacillus, Brevibacterium, Burkholderia, Methylobacillus,
Microbacterium, Novosphingobium, Paucibacter, Pseudomonas, Ralstonia, Rhizobium, Rhodococcus, Sphingomonas, Sphingopyxis, Sphingosinicella, Stenotrophomonas, and Trichaptum; Dziga et al. 2013, Kormas and Lymeropoulos 2013) suggests that these bacteria are of similar sensitivity to copper algaecide exposures as target cyanobacteria (i.e. *M. aeruginosa*). Pairing of bacterial analyses with analytical measures of MC-LR concentrations provides evidence that differences in MC-LR degradation between copper treatments were due to changes in bacterial communities.

The overall objective of this study was to determine the influence of copper concentrations and formulations on degradation of MC-LR following copper algaecide exposures. The specific objectives were to 1) measure rates and extents of MC-LR\textsubscript{Tot} degradation over time following exposures to a series of concentrations of three copper-based algaecides; 2) compare rates and extents of MC-LR degradation between copper concentrations (0, 0.1, 0.5, 1.0, and 5.0 mg Cu/L); 3) compare rates and extents of MC-LR degradation between copper formulations (CuSO\textsubscript{4}, Cutrine Plus\textsuperscript{®}, and Algimycin\textsuperscript{®}); and 4) measure and compare relative abundance and diversity of bacteria among treatments (formulation and concentration).
Methods

Algal Culture and Experimental Conditions

*M. aeruginosa* (CPCC 300) was used in this study because it is a consistent and reliable producer of MC-LR. *M. aeruginosa* was obtained from the Canadian Phycological Culture Center (CPCC) at the University of Waterloo in Waterloo, Ontario, Canada, and was cultured in ~35 L aquaria in BG-11 medium (Ripka et al. 1979). At the time of *M. aeruginosa* culture initiation, formulated BG-11 medium was amended with water collected from a pond in Anderson, SC where MC-LR producing cyanobacteria were present. Copper exposures were initiated when *M. aeruginosa* achieved a cell density of 4.5x10^6 cells/mL. Water characteristics of amended BG-11 medium with 4.5x10^6 cells of *M. aeruginosa* mL were: pH = 8.23, dissolved O_2 = 8.41 mg/L, conductivity = 845 µS/cm, alkalinity = 78 mg CaCO_3/L, hardness = 54 mg CaCO_3/L.

Temperature was maintained at 21±2°C with a 12:12 h light:dark cycle and light intensity of 1980-3340 LUX provided by cool-white fluorescent bulbs (Residential Ecolux 40 W, GE).

Following copper exposures, flasks were covered with Parafilm® and aerated using compressed air through airline tubing to maintain dissolved O_2 concentrations of approximately 8 mg O_2/L. Flasks were kept in the dark for the duration of the experiment to minimize photolysis of MC-LR (photolysis of MC-LR has been measured in the presence of water soluble pigments such as phycocyanin and phycoerythrin; Tsuji et al. 1994).
Copper Exposures

*M. aeruginosa* was transferred from stock culture into 250 mL acid washed borosilicate flasks and exposed to a series of concentrations of three copper algaecides (CuSO₄, Cutrine-Plus®, and Algimycin-PWF®; Table 2). Stock solutions of 1,000 mg Cu/L were prepared from each algaecide using Nanopure® water before initiation of toxicity experiments. Appropriate volumes of stock copper solutions were added to each flask to achieve targeted copper concentrations. Three replicate flasks were included for each copper exposure concentration. *M. aeruginosa* was exposed to four copper concentrations, 0.1, 0.5, 1.0, and 5.0 mg Cu/L as CuSO₄, Cutrine-Plus®, or Algimycin-PWF®. Untreated controls did not receive copper additions. Acid soluble copper concentrations were measured immediately following copper exposure to confirm targeted (nominal) exposures using an Agilent PSD 120 Flame Atomic Absorption Spectrometer (APHA 2012).

MC-LR Sampling

MC-LR was measured pre-treatment and following copper exposures to capture a minimum of three half-lives. MC-LRₐq and MC-LRₜot were measured directly before treatment and 12h, 24h, 36h, 48h, 72h, 96h, and 7d post-copper exposures. Flasks were mixed at each sampling time and 10mL of sample was pipetted from each beaker (5 mL for MC-LRₐq and 5mL for MC-LRₜot). MC-LRₐq was separated by filtering 5mL of sample from each flask through a Millipore® 0.45μm nitrocellulose filter. Filtrates were
then concentrated by water-bath evaporation (60-80°C) for approximately 8-12 h. Once
dry, concentrate was re-suspended in 1mL of 80:20 Nanopure® water and HPLC (high
performance liquid chromatography) grade acetonitrile, vortexed to mix, and transferred
to 2mL HPLC vials for HPLC quantification.

Collected whole samples (i.e. unfiltered) were evaporated and MC-LR\textsubscript{Tot} was
extracted from dried material. For MC-LR extraction, dried algal material was frozen at -
80°C, thawed, and re-suspended in 1 mL of 80:20 Nanopure® water and HPLC grade
acetonitrile. Re-suspended pellets were stored at 4°C overnight (approximately 12 h) in
solvent and then sonicated using a Branson® 5210 sonic bath for 5 minutes. Samples
were then centrifuged and filtered through a Millipore® 0.45µm nitrocellulose filter into
2 mL HPLC vials for MC-LR quantification.

**MC-LR Quantification**

MC-LR concentrations were measured using HPLC with ultraviolet detection
(Sangolkar et al. 2006). A Dionex Ultimate 3000® HPLC was utilized with an Acclaim
Polar Advantage II® column (5µm, 120Å, 4.6x250nm). Parameters for analysis were:
detection = 243 nm, temperature = 30°C, pressure = 1,500 psi, flow = 1 mL/min, and
injection volume = 60 µL. Solvents included Nanopure® H\textsubscript{2}O/ 0.1% trifluoroacetic acid
and HPLC grade acetonitrile. Peak area was measured at a detention time of 14.0-15.0
minutes and MC-LR concentrations were calculated by comparing measured area units to
known MC-LR standards (Beagle Bioproducts®, Columbus, Ohio). Standards of 100 µg
/L MC-LR were measured between every 20 samples and variability was calculated to
confirm measurement accuracy and precision. Method specific detection limits for MC-LR (lowest concentration at which the measured MC-LR concentration was significantly different from the solvent blank) (APHA 2005) was 2.0 µg MC-LR/L (including a 5-fold concentration factor) (ANOVA, α=0.05, p=0.0022).

**MC-LR Degradation**

MC-LR degradation extents (percent removal) and rates (slopes) calculated where the most rapid MC-LR degradation occurred during the course of the experiment were used to compare copper treatments (i.e. copper concentrations and formulations) in terms of MC-LR degradation. Extents of MC-LR degradation were calculated (Equation 1) and then compared between treatments at each sampling time. ANOVA was used to compare MC-LRTot concentrations between copper treatments at each sampling time (JMP v. 12, 2014).

Equation (1). \[ \text{Percent removal (\%) } = \frac{C_0 - C}{C_0} \times 100 \]

Where, initial concentration of MC-LR is denoted \([C_0]\) (mg/L) and \([C]\) (mg/L) is concentration of MC-LR at a specific sampling time.

Linear regression (JMP v. 12, 2014) and calculated 95% confidence intervals were used to compare slopes (rates) between treatments where the most rapid MC-LR degradation occurred (i.e. three sampling points from 36-72 h post-copper exposure at which greater than 90% of all MC-LR degradation occurred).
**Bacterial Sampling**

Bacterial samples were collected pre-treatment from the stock algal culture and 72 h following copper exposures. Sampling time was selected for bacterial analyses after copper exposures based on measured degradation rates of MC-LR, which were greatest between 36 and 72 h. Five milliliters of each treatment replicate (i.e. each copper algaecide concentration and formulation) were combined and mixed in a 30-mL medicine cup. Twelve milliliters of the mixed composite sample were filtered through a 0.45 µm Milipore® nitrocellulose filter. The filtrate was discarded and the filter was stored at -80ºC in a sterile, 15-mL DNAse/RNAse-free polypropylene Nalgene® container.

**Microbiome Sequencing and Analyses**

DNA was extracted from sample filters using the MO BIO Power Water DNA Isolation Kit. Targeted DNA sequencing was used to identify bacteria present in samples via polymerase chain reaction (PCR) amplification of the v3/v4 region of the 16S ribosomal RNA gene (Klindworth et al., 2013). Library preparation and sequencing were performed as per the manufacturer’s instructions for MiSeq v3 paired-end 300 bp sequencing (Illumina). Library preparation included positive and negative controls, with the former consisting of mock communities, and the latter where no DNA is added to the PCR, and the sample is carried through to sequencing. Sequencing data was processed by UPARSE to merge paired reads, perform quality filtering, and cluster operational taxonomic units (OTU) using a 97% identity threshold (Edgar, 2013). Primer sequences were removed prior to OTU clustering. Taxonomic classifications were performed by
UPARSE using the UTAX algorithm and the corresponding RDP reference data (v16). Weighted UniFrac distance measure (Lozupone et al., 2011) was used for ordination analyses using the “phyloseq” package (McMurdie and Holmes, 2013) in R. Bacterial alpha diversity (Simpson’s reciprocal index) was calculated with QIIME (Caporaso et al., 2010; Edgar, 2010).

Known MC-degrading bacteria were identified in sequencing results by BLAST analysis (Altschul et al., 1990) that compared representative sequences for each OTU to 16S rRNA gene sequences from published studies identifying degradation (Dziga et al., 2013). Organisms that have been described in literature as MC-degrading but that did not have 16S rRNA gene sequence accession information were not queried for in the sequencing results.
Results

*M. aeruginosa Responses and Copper Exposures*

*M. aeruginosa* populations declined following experiment initiation (i.e. copper exposures, aeration, and movement away from light) in both treatments and untreated controls. Objectives of the present experiment were to compare relative influences of copper concentrations and formulations on degradation of MC-LR as a result of copper exposures to heterotrophic MC-degrading bacteria. Responses of *M. aeruginosa* to copper exposures were not a primary consideration in this study, therefore *M. aeruginosa* viability was not necessary to achieve experimental objectives. Following *M. aeruginosa* population decline, MC-LR$_{Aq}$ was measured to determine extent of MC-LR$_{Cell}$ release to MC-LR$_{Aq}$. Pretreatment MC-LR$_{Tot}$ concentrations were 87±5 µg MC-LR/L with approximately 15±2% of MC-LR$_{Tot}$ present as MC-LR$_{Aq}$. Within 24h post-copper exposure, MC-LR$_{Aq}$ accounted for 88±10% of MC-LR$_{Tot}$ in copper exposures and in untreated controls.

Measured pretreatment copper concentration was approximately 20 µg/L. Targeted copper exposures (0.1, 0.5, 1.0, and 5.0 mg Cu/L) in algaecide treatments were analytically confirmed and percent error between measured and targeted (nominal) copper exposures was 4-32%, 2-32%, and 8-16% for CuSO$_4$, Cutrine-Plus®, and Algimycin-PWF®, respectively. Statistical analyses (i.e. ANOVA) to compare treatments utilized nominal (i.e. targeted) concentrations.
**MC-LR Degradation**

MC-LR$_{Tot}$ concentrations declined precipitously between 36 and 72h post-copper exposures, with 90-97% decreases in MC-LR$_{Tot}$ concentrations in untreated controls, 0.1, 0.5, and 1.0 mg Cu/L exposures (Figure 4.1; Table 4.3). Seven days after copper exposures, MC-LR$_{Tot}$ concentrations decreased 61±47%, 29±1%, and 27±4% following exposure to 5.0 mg Cu/L as CuSO$_4$, Cutrine-Plus®, and Algimycin-PWF®, respectively (Table 4.3). For all copper algaecide formulations tested, MC-LR$_{Tot}$ concentrations were below method detection (2 µg MC-LR/L) within 96h following exposure to copper concentrations of 0.1, 0.5, and 1.0 mg Cu/L. MC-LR$_{Tot}$ concentrations in untreated controls were also below detection within in 96h. Following exposure to 5.0 mg Cu/L, MC-LR$_{Tot}$ concentrations were detectable throughout the experiment (7d). MC-LR$_{Tot}$ concentrations were 35±43, 64±2, and 64±5 µg MC-LR/L (compared to pretreatment concentrations of 87±5 µg MC-LR/L) 7d following exposure to 5.0 mg Cu/L as CuSO$_4$, Cutrine-Plus®, and Algimycin-PWF®, respectively.

Rates of MC-LR$_{Tot}$ degradation calculated using linear regression between 36-72h post-copper exposures were similar (based on comparisons of 95% confidence intervals) among untreated controls and copper exposures of 0.1, 0.5, and 1.0 mg Cu/L. Rates of MC-LR$_{Tot}$ degradation following exposure to 5.0 mg Cu/L were calculated using linear regression between 0-168h post-copper exposure. Calculated rates for 0.1-1.0 mg Cu/L treatments and controls ranged from -1.7 to -2.2 µg MC-LR$_{Tot}$/L hr$^{-1}$ (Table 4.4). In contrast, following exposure to the highest tested copper concentration (5.0 mg Cu/L),
rates of MC-LR\textsubscript{Tot} degradation were significantly less than all other copper treatments and the untreated control and were -0.27, -0.14, and -0.11 µg MC-LR\textsubscript{Tot}/L hr\textsuperscript{-1} for CuSO\textsubscript{4}, Cutrine-Plus\textsuperscript{®}, and Algimycin-PWF\textsuperscript{®}, respectively (Figure 4.1; Table 4.4).

Among all copper formulations tested and untreated controls, MC-LR degradation was comparable in terms of rates and extents (Figure 4.1, Table 4.3). MC-LR\textsubscript{Tot} concentrations in each treatment concentration (i.e. control, 0.1, 0.5, 1.0, and 5.0 mg Cu/L) at 24, 36, 48, 72, 96, and 168h post-copper exposure were not significantly different between copper formulations (α=0.05, p=0.055-0.94). Rates were also not significantly different between formulations (based on 95% confidence intervals; Table 4.4).

**Bacterial Analyses**

**Bacterial Community Composition**

Bacteria identified in genetic sequencing results from treatments in this study which have been identified as capable of MC degradation (based on literature review) include *Novosphingobium, Rhizobium gallicum, Sphingomonas, Sphingopyxis,* and *Sphingosinicella microcystinivorans*. Bacterial diversity decreased following exposure to copper concentrations greater than 0.1 mg Cu/L (Simpson’s reciprocal diversity index, Table 4.5). Bacterial communities in untreated controls and 0.1 mg Cu/L treatments were similar to one another, but differed from all other copper exposures (0.5-5.0 mg Cu/L) (Figure 4.2, Table 4.5) in terms of diversity and community composition. Bacterial community compositions were similar among 0.5-5.0 mg Cu/L treatments (Figure 4.2;
Table 4.5). The subset of MC degrading organisms in the control and 0.1-0.5 mg Cu/L treatments are similar to one another and the 1.0 and 5.0 mg Cu/L treatments are similar to one another (Figure 4.3). *Sphingosinicella microcystinivorans* decreases in relative abundance with increasing copper concentrations, while *Sphingomonas* and *Sphingopyxis* increase in relative abundance.
Discussion

Following copper exposures, *M. aeruginosa* populations declined in untreated controls and treatments. Decline of *M. aeruginosa* in untreated controls was likely due to changes in environmental conditions (i.e. aeration and movement away from light; Chen et al. 2009). In this study, the purpose of untreated controls was to measure MC-LR degradation in the absence of amended copper. Therefore, decline of *M. aeruginosa* in untreated controls provided an advantageous opportunity for direct comparison of MC-LR<sub>Tot</sub> degradation rates between untreated controls and copper exposures, rather than relying on positive controls (i.e. MC-LR standard amended to untreated, live *M. aeruginosa*). Decline of *M. aeruginosa* controls also provided an opportunity to measure microcystin release from naturally declining *M. aeruginosa*, which resulted in release of over 90% of MC-LR<sub>Tot</sub> to the aqueous phase.

Results of the present study indicate that copper concentration can influence degradation of MC-LR<sub>Tot</sub>. However, exposures to copper concentrations registered for use (i.e. 0.1-1.0 mg Cu/L as Cutrine-Plus<sup>®</sup> and Algimycin-PWF<sup>®</sup>) did not influence MC-LR<sub>Tot</sub> degradation compared to untreated controls. MC-LR<sub>Tot</sub> degradation in untreated controls and copper exposures between 0.1 and 1.0 mg Cu/L occurred primarily between 36 and 72 h. Degradation began following release of MC-LR to MC-LR<sub>Aq</sub> 24 h post-copper exposure. The decrease in MC-LR<sub>Tot</sub> concentrations between 24 and 36 h was less rapid than between 36 and 72 h, presumably due to time required for MC-degrading bacteria to increase in density once MC-LR was available following release from *M.*
*aeruginosa* cells. The highest copper concentrations tested (5.0 mg Cu/L) resulted in persistence of MC-LR$_{Tot}$ in comparison to other copper exposures (0.1-1.0 mg Cu/L) and untreated controls. As MC-LR fate is predominately regulated by bacterial degradation, persistence of MC-LR$_{Tot}$ at 5.0 mg Cu/L compared to other copper treatments was presumably due to changes in bacterial communities, specifically bacteria capable of MC-LR degradation.

Bacteria present in this study previously identified as capable of MC-degradation include *Novosphingobium, Rhizobium gallicum, Sphingomonas, Sphingopyxis,* and *Sphingosinicella microcystinivorans* (Dziga et al. 2013, Kormas and Lymperopoulou 2013). Although diversity of bacteria was greater in untreated controls and 0.1 mg Cu/L exposures compared to 0.5, 1.0, and 5.0 mg Cu/L exposures, changes in bacterial diversity did not manifest as differences in bacterial degradation. The relative abundance of *Novosphingobium, Sphingomonas,* and *Sphingopyxis* increased following exposure to 5.0 mg Cu/L, which suggests these MC-degrading bacteria may be less sensitive to copper than other MC-degrading bacteria (i.e. *Sphingosinicella microcystinivorans*), as well as other bacteria as overall diversity decreased. However, despite the robustness of MC-degrading bacteria following exposure to copper concentrations of 5.0 mg Cu/L, extents of MC-LR degradation following copper of exposures of 5.0 mg Cu/L decreased. These results suggest that densities of MC-degrading bacteria likely decreased, resulting in decreased MC-LR degradation. However, densities of bacteria were not confirmed in this study.
Although copper concentration affected degradation of MC-LR, the formulation of copper algaecide did not influence extents or rates of MC-LR degradation, nor did it influence diversity of bacteria present following treatment. This suggests that in the present study, copper form (CuSO₄, copper-ethanolamine, and copper-citrate and gluconate) did not alter copper exposures to bacteria to an extent that affected MC-LR degradation.
Conclusions

Results of the present study indicate that copper concentrations can influence degradation rates of MC-LR following copper-algaecide exposures. However, exposures to copper concentrations registered for use (i.e. 0.1-1.0 mg Cu/L as Cutrine-Plus® and Algimycin-PWF®) did not influence MC-LR\textsubscript{Tot} degradation compared to untreated controls. Certain MC-degrading bacteria increased in proportion following exposure to increasing copper concentrations, while others decreased. This suggests that some MC-degrading bacteria may be less sensitive than others to copper algaecides. Overall, bacterial diversity is lower at treatment concentrations above 0.1 mg Cu/L, suggesting that non-MC degrading heterotrophic bacteria are also affected by copper algaecides. Copper formulation did not significantly alter degradation rates or bacterial composition at comparable copper exposures. These data provide a more thorough understanding of the influences of copper algaecides on MC-LR degradation over time, and can be used to inform more accurate risk evaluations and use of copper-based algaecides for management of MC-LR producing \textit{M. aeruginosa}. 

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References


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<table>
<thead>
<tr>
<th>Physical, chemical, and biological characteristics of MC-LR</th>
<th></th>
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</thead>
<tbody>
<tr>
<td>Microcystin-LR (L – leucine, R – arginine)</td>
<td></td>
</tr>
<tr>
<td><strong>Formula</strong></td>
<td>$\text{C}<em>{49}\text{H}</em>{74}\text{N}<em>{10}\text{O}</em>{12}$</td>
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<td><strong>Color/ Form</strong></td>
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<tr>
<td><strong>Photolysis (half-life)</strong></td>
<td>$&gt;26$ days (13.9 $\mu$E m$^{-2}$ s$^{-1}$ fluorescent illumination and natural sunlight)$^a$</td>
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<tr>
<td></td>
<td>$&lt;10$ min (1.5-25.5 W m$^{-2}$ UVR)$^b$</td>
</tr>
<tr>
<td><strong>Hydrolysis</strong></td>
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<tr>
<td><strong>Bacterial Aerobic Degradation (half-life)</strong></td>
<td>4-14 days$^c$, 1.2-7.7 days$^d$</td>
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<tr>
<td><strong>Solubility in Water</strong></td>
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<td><strong>Boiling point ($^\circ$C)</strong></td>
<td>Structurally resistant to 300$^\circ$C$^e$</td>
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<tr>
<td><strong>Log$\text{K}_{\text{ow}}$</strong></td>
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<tr>
<td><strong>Sorption Extent (%)</strong></td>
<td>33$^f$, 81$^h$, 24-44$^i$</td>
</tr>
<tr>
<td><strong>$K_d$ (L kg$^{-1}$) (Sediment)</strong></td>
<td>5-35$^f$, 0.23-6.95$^f$, 1-13$^i$</td>
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</tbody>
</table>

$^a$ Tsuji et al. 1994  
$^b$ Tsuji et al. 1995  
$^c$ Edwards et al. 2008, WHO 2003  
$^d$ Chen et al. 2008  
$^e$ Rivasseau et al. 1998  
$^f$ Wu et al. 2011  
$^g$ Miller et al. 2001  
$^h$ Morris et al. 2000  
$^i$ Calculated from Munusamy et al. 2012 (Fig. 2, pg. 2395)  
$^i$ Mohamed et al. 2007
Table 4.2 Characteristics of copper sulfate pentahydrate (CuSO₄) and Cutrine-Plus®.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>CuSO₄•5H₂O</th>
<th>Cutrine-Plus®</th>
<th>Algimycin-PWF®</th>
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<tr>
<td>Active ingredient</td>
<td>Copper</td>
<td>Copper</td>
<td>Copper</td>
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<tr>
<td>% Active ingredient</td>
<td>23.7</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Maximum label concentration as copper</td>
<td>2 mg/L&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1 mg/L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 mg/L&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Formulation</td>
<td>Copper sulfate pentahydrate</td>
<td>Copper ethanolamine complex&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Copper citrate and gluconate&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Appearance</td>
<td>Blue crystals</td>
<td>Blue liquid</td>
<td>Blue-green liquid</td>
</tr>
<tr>
<td>Water solubility</td>
<td>415,997 mg/L</td>
<td>Miscible&lt;sup&gt;*&lt;/sup&gt;</td>
<td>Miscible&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Boiling point (°C)</td>
<td>106&lt;sup&gt;**&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific gravity (g/cm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>1.21&lt;sup&gt;**&lt;/sup&gt;</td>
<td>1.22</td>
<td>1.25</td>
</tr>
<tr>
<td>pH</td>
<td>3.22&lt;sup&gt;**&lt;/sup&gt;</td>
<td>10.3-10.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.7-1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Physical and chemical characteristics are of the original compound unless otherwise noted.

* Can mix with water in all proportions.

** Physical and chemical characteristics of saturated solution

<sup>a</sup> Applied Biochemists product label
<sup>b</sup> Applied Biochemists MSDS
<sup>c</sup> Murray-Gulde et al. 2002
<sup>e</sup> Chem One Ltd. product label
Table 4.3 Percent MC-LR<sub>Tot</sub> degradation from initial MC-LR<sub>Tot</sub> concentrations following copper algaecide exposures

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CuSO&lt;sub&gt;4&lt;/sub&gt; (mg Cu/L)</th>
<th>Cutrine-Plus&lt;sup&gt;®&lt;/sup&gt; (mg Cu/L)</th>
<th>Algimycin&lt;sup&gt;®&lt;/sup&gt; (mg Cu/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.1 0.5 1.0 5.0</td>
<td>0.1 0.5 1.0 5.0</td>
<td>0.1 0.5 1.0 5.0</td>
</tr>
<tr>
<td>24 HAT</td>
<td>2±4</td>
<td>6±5 12±9  5±6 17±7</td>
<td>12±8 14±10 10±2 16±1</td>
<td>20±3 12±5 10±5 13±5</td>
</tr>
<tr>
<td>36 HAT</td>
<td>15±4</td>
<td>17±6 19±6 14±10 *</td>
<td>15±10 20±5  6±6  *</td>
<td>23±2 21±4 14±6 *</td>
</tr>
<tr>
<td>48 HAT</td>
<td>34±6</td>
<td>39±10 46±8 53±30 22±1</td>
<td>31±13 39±4  29±4 14±3</td>
<td>37±2 41±7 30±6 25±1</td>
</tr>
<tr>
<td>72 HAT</td>
<td>99±1</td>
<td>99±1 98±1 96±2 *</td>
<td>99±1 96±3 94±1 *</td>
<td>98±2 93±6 91±4 *</td>
</tr>
<tr>
<td>96 HAT</td>
<td>BDL</td>
<td>BDL BDL BDL 21±7</td>
<td>BDL 98±3 BDL 25±2</td>
<td>BDL BDL BDL 19±5</td>
</tr>
<tr>
<td>168 HAT</td>
<td>BDL</td>
<td>BDL BDL BDL 61±48</td>
<td>BDL BDL BDL 30±1</td>
<td>BDL BDL BDL 27±4</td>
</tr>
</tbody>
</table>

*No data collected for MC-LR concentration at sampling time. HAT=hours after copper treatment. BDL=below method detection limit (i.e. 2 µg MC-LR/L)
Table 4.4 MC-LR<sub>Tot</sub> degradation rates 36-72 h following CuSO<sub>4</sub> (a), Cutrine-Plus® (b), and Algimycin-PWF® (c) exposures

<table>
<thead>
<tr>
<th>Copper (mg/L)</th>
<th>Copper Sulfate</th>
<th>Cutrine-Plus®</th>
<th>Algimycin-PWF®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k (95% CI)</td>
<td>R²</td>
<td>k (95% CI)</td>
</tr>
<tr>
<td>0</td>
<td>-2.1 (-2.4, -1.8)</td>
<td>0.98</td>
<td>-2.1 (-2.4, -1.8)</td>
</tr>
<tr>
<td>0.1</td>
<td>-1.9 (-2.2, 1.7)</td>
<td>0.98</td>
<td>-1.9 (-2.3, -1.5)</td>
</tr>
<tr>
<td>0.5</td>
<td>-2.1 (-2.3, 2.0)</td>
<td>0.98</td>
<td>-2.0 (-2.2, 1.7)</td>
</tr>
<tr>
<td>1.0</td>
<td>-1.7 (-2.4, -1.0)</td>
<td>0.84</td>
<td>-2.2 (-2.4, -1.9)</td>
</tr>
<tr>
<td>5.0</td>
<td>-0.27 (-4.3, -0.10)</td>
<td>0.54</td>
<td>-0.14 (-0.18, -0.10)</td>
</tr>
</tbody>
</table>

Rates (k; µg MC-LR<sub>Tot</sub>/L hr<sup>−1</sup>), confidence intervals (CI), and R<sup>2</sup> values were calculated with linear regression using 3 sampling points (MC-LR<sub>Tot</sub> concentrations) between 36 and 72h post-copper exposure for copper concentrations between 0 and 1.0 mg Cu/L, and 4 sampling points between 0 and 168h were used for linear regression analysis of MC-LR<sub>Tot</sub> concentrations over time following exposure to 5.0 mg Cu/L.
Table 4.5 Bacterial composition (diversity and proportion of MC-degraders) 72 h post-copper exposures

<table>
<thead>
<tr>
<th>Copper (mg/L)</th>
<th>Copper Sulfate</th>
<th>Cutrine-Plus®</th>
<th>Algimycin-PWF®</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Simpson’s Reciprocal (Diversity Index)</td>
<td>Proportion of MC- Degrading Bacteria</td>
<td>Simpson’s Reciprocal (Diversity Index)</td>
</tr>
<tr>
<td>0</td>
<td>27</td>
<td>3.30%</td>
<td>27</td>
</tr>
<tr>
<td>0.1</td>
<td>16</td>
<td>2.60%</td>
<td>26</td>
</tr>
<tr>
<td>0.5</td>
<td>9</td>
<td>0.48%</td>
<td>8</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>0.52%</td>
<td>9</td>
</tr>
<tr>
<td>5.0</td>
<td>11</td>
<td>1.35%</td>
<td>16</td>
</tr>
</tbody>
</table>

Values presented in Table 4.5 represent bacterial communities present 72 HAT (hours after copper treatment). Simpson’s reciprocal index values represent diversity of total bacterial communities in each treatment and % MC-degraders represents the proportion of the total bacterial community composed of known MC-degrading organisms (based on published literature).
Figure 4.1 MC-LR$_{tot}$ concentrations over time following CuSO$_4$ (a), Cutrine-Plus$^\text{®}$ (b), and Algimycin-PWF$^\text{®}$ (c) exposures. n=3, error bars= 1 standard deviation
Figure 4.2 Non-metric multidimensional scaling (NMDS) plot of weighted Unifrac distances to compare bacterial communities among copper treatments (concentrations and formulations) and controls. Ordination of bacterial community composition between copper concentrations and formulations, and controls displays differences between treatments. Each point represents a composite of sample replicates (n=3) of each treatment and control. The relative distance between samples reflects relative differences and similarities in composition of bacterial communities in each sample. Alg=Algimycin-PWF®, Cu-Plus=Cutrine-Plus®
Figure 4.3 Relative abundance of MC-degrading bacteria present in treatments 72 h post-copper exposure. A pre-treatment sample was taken before copper addition.
CHAPTER 5
MICROCYSTIN-LR RELEASE AND DEGRADATION FOLLOWING EXPOSURES OF *MICROCYSTIS* TO A CHELATED COPPER ALGAECIDE

Abstract

Persistence of the cyanobacterial produced hepatotoxin, microcystin (MC), is sometimes cited as a potential consequence of copper-algaecide applications. This study used in situ mesocosms to test the relationship between copper concentrations and MC-LR degradation rates and extents in a controlled field setting. The MC-LR-producing cyanobacterium *Microcystis* was exposed to a series of copper concentrations (0.4, 0.8, and 2.0 mg Cu/L) of a chelated copper algaecide. Rates and extents of MC-LR degradation, as well as bacterial diversity and density, were measured and compared between copper treatments (i.e. concentrations). Results indicated that copper concentration influenced MC-LR degradation and bacterial density, diversity, and relative abundance of MC-degrading bacteria. However, increased copper concentrations did not always result in slower decline in MC-LR\textsubscript{Aq} concentrations as previously hypothesized. Slowest decline in MC-LR concentrations occurred in the lowest copper exposures (0.4 mg Cu/L), at a rate of -6.8 µg MC-LR/L d\(^{-1}\). However, this was likely due to slower post-treatment decline of *Microcystis* rather than copper effects on bacterial degradation as densities of bacteria were similar between untreated controls and 0.4 mg Cu/L exposures. At the two highest copper concentrations, 2.0 mg Cu/L exposures resulted in significantly slower MC-LR degradation rates than 0.8 mg Cu/L exposures, -
16.6 µg MC-LR/L d⁻¹ and -10.8 µg MC-LR/L d⁻¹, respectively. However, significant differences in degradation rates manifested as only a 1.5 d difference in MC-LR removal times (i.e. 48 h vs. 84 h). Bacteria density was not different between 0.8 and 2.0 mg Cu/L exposures, however it was an order of magnitude less than bacteria density measured following 0.4 mg Cu/L exposures and in untreated controls. Results of this study indicate that copper from algaecide applications can influence bacterial assemblages as well as rates of MC-LRₐq degradation. However, in this study copper exposures 2x the allowable concentration of the tested algaecide (2.0 mg Cu/L) resulted in >97% MC-LR degradation in less than 4d. Based on data from the present study, typical copper algaecide applications (i.e. ≤1.0 mg Cu/L) will likely not result in persistence of MC-LR concentrations.
Introduction

Microcystins (MCs) are cyclic hepatapeptides produced by cyanobacteria genera such as *Anabaena, Anabaenopsis, Hapalosiphon, Microcystis, Nostoc, Oscillatoria*, and *Planktothrix* (Graham et al. 2008). There are over 80 structural variants of MCs, and microcystin-LR (MC-LR) is among the most problematic and toxic of the MC congeners (WHO 2003a). MC-LR is an endotoxin that is contained within the cyanobacterial cell as cellular MC-LR (MC-LR_{Cell}) until cell death upon which it is released into surrounding water as aqueous MC-LR (MC-LR_{Aq}) (Zohary and Pais Madeira 1990, Watanabe et al. 1992, Chorus and Bartram 2000). MC-LR in either form (i.e. MC-LR_{Aq} or MC-LR_{Cell}) can present ecological and human health risks (WHO 2003a, Zurawell et al. 2005, EPA 2015) and interfere with designated uses of freshwater resources including recreation (i.e. beach and lake closures; WHO 2003b, EPA 2016), livestock and wildlife management and propagation (i.e. poisonings of fish, water fowl, domestic pets, and livestock; Carmichael et al. 1989 and 2001), and potable water use (AWWA 2015, EPA 2012).

Alleviation of problems associated with noxious algal blooms can be achieved by decreasing densities of problematic MC-LR-producing cyanobacteria through use of algaecides (Gettys et al. 2014). Copper-based algaecides are used to manage noxious algae and restore water resources to their intended uses (Huddleston et al. 2016, Gettys et al. 2014). However, use of copper-based algaecides is sometimes limited in treatment of MC-LR-producing cyanobacteria due to concerns regarding potential release of intracellular MC-LR (MC-LR_{Cell}) to surrounding water upon copper treatment, as well as
potential persistence of MC-LRAq following release (e.g. Jones and Orr, 1994). Effects of copper algaecides on MC-LR release have been extensively studied (Kenefick et al. 1993, Jones and Orr 1994, Touchette et al. 2008, Polyak et al. 2013, Zhou et al. 2013, Fan et al. 2013 and 2014, Greenfield et al. 2014, Tsai et al. 2015, Iwinski et al. 2016a, Iwinski et al. 2016b in review). However, MC-LR persistence due to copper algaecide exposures has been relatively unstudied, despite potential influence on management decisions for MC-LR-producing cyanobacteria.

The hypothesis of MC-LR persistence following copper algaecide exposures is based on the assumption that copper-algaecides can decrease targeted cyanobacteria densities as well as densities of resident heterotrophic bacteria that are responsible for MC-LR degradation. MC-LR is a relatively stable compound in aquatic systems and is typically resistant to removal and/or degradation from hydrolysis, photolysis, sorption, and oxidation (i.e. half-lives from weeks to years; Tsuji et al. 1994, Rivasseau et al. 1994, WHO 2003a, Schmidt et al. 2014). The primary and most rapid degradation pathway for MC-LR is bacterial degradation with half-lives from <1 day to weeks (Edwards et al. 2008, Chen et al. 2008). Therefore, effects of copper algaecides on MC-LR degrading bacteria could hypothetically influence MC-LR fate (i.e. biodegradation).

Evidence in support of MC-LR persistence following algaecide exposures exists primarily in a single field study where a copper algaecide treatment of an unspecified concentration resulted in persistence of MC-LR in a drinking water reservoir for approximately 21 days (Jones and Orr, 1994). A recent laboratory study tested the
influence of different copper concentrations from algaecide applications on MC-LR degradation (Iwinski et al. 2017, in review). Iwinski et al. (2016, in review) did not measure changes in MC-LR degradation in the range of copper concentrations that can be applied as copper algaecides in the United States (i.e. 0.1-1.0 mg Cu/L). However, decreased MC-LR degradation rates were measured following exposure to copper concentrations of 5.0 mg Cu/L (five times the allowable application of most algaecides). In this study, field confirmation of copper concentration dependent degradation of MC-LR was accomplished using in situ mesocosms and measuring changes in MC-LR degradation rates and microbial communities following copper algaecide applications. Mesocosm studies incorporated field parameters such as sediments and climatic conditions, while allowing for adequate replication and manipulation for testing multiple copper concentrations.

The overall objective of this study was to determine the influence of copper concentration on MC-LR degradation following copper algaecide exposures. The specific objectives to support the overall objective were to 1) measure responses of *M. aeruginosa* to targeted concentrations of Cutrine-Plus® in terms of chlorophyll *a* and cell density, 2) measure and compare concentrations of MC-LR$_{\text{Tot}}$ and MC-LR$_{\text{Aq}}$ following exposure to a series of copper concentrations, 3) measure and compare rates and extent of MC-LR$_{\text{Tot}}$ and MC-LR$_{\text{Aq}}$ degradation over time following exposure to a series of copper concentrations, and 4) measure and compare bacterial density, relative abundance, and diversity following exposure to a series of copper concentrations.
Material and Methods

Field Site and Enclosures

The study was conducted in a pond with a surface area of approximately 4,000 m² (~1 acre) and an average depth of approximately 1.2 m (4 ft). The pond was located at TRI Environmental, Inc.’s Denver Downs Research Facility in Anderson, SC. The pond received both agricultural and livestock runoff from the surrounding 900-acre farm (Table 5.1). High-density polyethylene, 30-gallon drums were modified and utilized as enclosures within the pond. The bottoms of the drums were removed and the open-ended drum (termed “enclosure”) was placed into the pond and secured into pond sediments (~5-10 cm into sediments), capturing algae within. Algae were then added or removed from individual enclosures based on visual observations to achieve similar cell densities among the enclosures. Initial cell densities within enclosures ranged from ~9.0x10⁵-2.4x10⁶ cells/ mL (Table 5.1). *M. aeruginosa* was the dominant alga in the pond (representing >99% of the algal assemblage) with *Euglena sp.* and *Anabaena sp.* present (representing <1% of the algal assemblage).

Copper Exposures

Copper exposures in mesocosms were prepared by estimating the volume of water within each enclosure using a cylindrical segment equation (Equation 1). Enclosures (cylinders) were inserted into sloped sediments (44.3±4.6° slope) of the pond littoral zone. Height from the sediment surface to the water surface was measured at the top (h₁)
and bottom (h₂) of the sloped sediments within each enclosure. The average volume (V) calculated for the 12 enclosures was 49±7 liters.

Equation 1. \[ V = \frac{1}{2} \pi r^2 (h_1 + h_2) \]

Cutrine-Plus® (Table 5.2) was then volumetrically measured for each enclosure using a 1,000 mg Cu/L stock solution of Cutrine-Plus® in Nanopure® water. Target exposure concentrations were 0.4, 0.8, and 2.0 mg Cu/L as Cutrine-Plus® with 3 replicate enclosures per concentration (n=3, N=12). Algaecide was then added to each enclosure and mixed thoroughly. Acid soluble copper concentrations were measured at initiation of the experiment to confirm exposures using a Perkin Elmer Optima 3100RL inductively coupled plasma-optical emission spectrometer (ICP-OES) with autosampler (APHA 2012). Mesocosms were aerated (Marine Metal Products, Bubble Box™ Portable Air Pump) to maintain dissolved oxygen concentrations above 8 mg/L in order to eliminate lack of oxygen as a potential factor in differences in aerobic bacterial degradation between copper treatments.

**Sampling Methods**

Samples were collected from each enclosure for measurements of MC-LR concentrations, chlorophyll a concentrations, and cell densities. Water in enclosures was mixed prior to sampling to evenly distribute *Microcystis* as the cyanobacteria otherwise formed a thin layer of cells on the water surface. Three 50 mL samples were collected from each enclosure; 20 mL were used for MC-LRₜₒₜ analysis, 30-50 mL were filtered for MC-LRₐₚ analysis (final volume of filtrate was recorded), and 5 mL were used for
chlorophyll \( a \) analysis. MC-LR samples were collected pre-treatment and every 12h for 7d following copper exposures. Samples for \textit{Microcystis} viability were collected pre-treatment and 5d after copper exposures.

\textit{Microcystis Responses}

Chlorophyll \( a \) was measured using a spectrophotometric method (APHA 2012) with a SpectraMax M2 spectrofluorometer (Molecular Devices Corp, Sunnyvale, CA). Chlorophyll \( a \) standards were acquired from Sigma Aldrich (C6144). Cell density was measured using light microscopy and an improved Neubauer hemocytometer (APHA 2016; Hausser Scientific Co. Horsham, PA 19044). \textit{Microcystis} was sonicated (Fisher Scientific, Sonic Dismembrator 550) for approximately five seconds prior to counting in order to disaggregate colonial cells for more accurate enumeration.

\textit{MC-LR Extraction and Concentration}

MC-LR\textsubscript{Aq} was separated by filtering approximately 50 mL of sample from each enclosure through a Millipore\textsuperscript{®} 0.45\(\mu\)m nitrocellulose filter. Total volumes of filtered field samples were recorded for calculation of MC-LR\textsubscript{Aq} concentrations. Filtrate was then concentrated by evaporation to dryness (using a water bath and compressed air) for approximately 8-12 hours. The concentrate was then re-suspended in 1 mL of 80:20 Nanopure\textsuperscript{®} water and HPLC grade acetonitrile, vortexed to mix, and transferred to 2 mL HPLC vials for HPLC quantification.

For MC-LR\textsubscript{Tot} quantification and extraction 20 mL of whole, unfiltered sample was evaporated to dryness, frozen at -80\(^\circ\)C, thawed, evaporated, and re-suspended in 1
mL of 80:20 Nanopure® water and HPLC grade acetonitrile. Re-suspended pellet was stored at 4°C overnight (approximately 12 hours) in solvent and then sonicated using a Branson® 5210 sonic bath for 5 minutes. Samples were then centrifuged and filtered through a Millipore® 0.45µm nitrocellulose filter into 2 mL HPLC vials for MC-LR_{Tot} quantification.

**MC-LR Quantification**

MC-LR concentrations were measured using high performance liquid chromatography (HPLC) with ultraviolet detection (Sangolkar et al. 2006). A Dionex Ultimate 3000® HPLC was utilized with an Acclaim Polar Advantage II® column (5µm, 120Å, 4.6x250nm). Parameters for analysis were: detection = 243 nm, temperature = 30°C, pressure = 1,500 psi, flow = 1 mL/min, and injection volume = 60 µL. Solvents included Nanopure® H₂O/ 0.1% trifluoroacetic acid and HPLC grade acetonitrile. Peak area was measured at a detention time of 14.0-15.0 minutes and MC-LR concentrations were calculated by comparing measured area units to known MC-LR standards (Beagle Bioproducts®, Columbus, Ohio). Known 100 µg MC-LR/L standards were measured between every 20 samples and variability was calculated to confirm measurement accuracy and precision. Method specific detection limits for MC-LR was 1.0 µg MC-LR/L (ANOVA, α=0.05, p=0.0022). With incorporation of concentration factors, detection limits were 0.5 µg MC-LR/L for MC-LR_{Tot} (based on 20x concentration factor) and 0.2-0.5 µg MC-LR/L for MC-LR_{Aq} (based on volume of final filtrate, i.e. 20-50 mL = 20-50x concentration factor).
**Extent of Removal and Degradation Rates**

Extent of removal of MC-LR concentrations, cell densities, and chlorophyll $a$ concentrations following copper exposures were calculated (Equation 2).

**Equation 2.**  
\[
\% \Delta = \left( \frac{C - C_0}{C_0} \right) \times 100
\]

In Equation 2, C indicates the measured MC-LR concentration, cell density, or chlorophyll $a$ concentration at a specific sampling time. $C_0$ is the initial pre-treatment microcystin concentration or *M. aeruginosa* viability parameter (i.e. cell density or chlorophyll $a$ concentration) in untreated controls. $\% \Delta$ indicates the percent change (i.e. % removal) from either pre-treatments values (MC-LR) or from untreated controls (chlorophyll $a$ and cell density).

MC-LR$_{Tot}$ and MC-LR$_{Aq}$ degradation following copper treatments (i.e. copper concentrations) were compared based on MC-LR extent of removal at each 12h sampling time. MC-LR degradation rates were calculated using linear regression for slopes where $>87-95\%$ of MC-LR degradation occurred and where lowest measured MC-LR concentrations plateaued (i.e. no longer decreased with time). MC-LR degradation rates were compared between copper treatments using calculated 95% confidence intervals (JMP v. 12, 2012).

**Bacterial Sampling**

Bacterial samples were collected from each mesocosm between 24-96 hours post-copper exposure. Samples selected for bacterial analyses after copper exposures were based on measured degradation rates of MC-LR, which were greatest between 36 and 72
hours. 50 mL of sample were collected in duplicate (i.e. 100 mL of total sample) from each mesocosm. Each 50 mL sample was filtered through a 0.45 µm Milipore® nitrocellulose filter. The filtrate was discarded and the filter was stored at -80°C in a sterile, 15-mL DNAse/RNase-free polypropylene Nalgene® container.

**Microbiome Sequencing and Analyses**

DNA was extracted from sample filters using the MO BIO Power Water DNA Isolation Kit. Targeted DNA sequencing was used to identify bacteria present in samples via polymerase chain reaction (PCR) amplification of the v3/v4 region of the 16S ribosomal RNA gene (Klindworth et al., 2013). Library preparation and sequencing were performed as per the manufacturer’s instructions for MiSeq v3 paired-end 300 bp sequencing (Illumina). Library preparation included positive and negative controls, with the former consisting of mock communities, and the latter where no DNA is added to the PCR, and the sample is carried through to sequencing. Sequencing data were processed by UPARSE to merge paired reads, perform quality filtering, and cluster operational taxonomic units (OTU) using a 97% identity threshold (Edgar, 2013). Primer sequences were removed prior to OTU clustering. Taxonomic classifications were performed by UPARSE using the UTAX algorithm and the corresponding RDP reference data (v16). Weighted UniFrac distance measure (Lozupone et al., 2011) was used for ordination analyses using the “phyloseq” package (McMurdie and Holmes, 2013) in R. Bacterial alpha diversity (Simpson’s reciprocal index) was calculated with QIIME (Caporaso et al., 2010; Edgar, 2010).
Known MC-degrading bacteria were identified in sequencing results by BLAST analysis (Altschul et al., 1990) that compared representative sequences for each OTU to 16S rRNA gene sequences from published studies identifying degradation (Dziga et al., 2013). Organisms that have been described in literature as MC-degrading but that did not have 16S rRNA gene sequence accession information were not queried for in the sequencing results.

**Bacteria Density: Most-probable number assay**

Total heterotrophs (grown with YTS250 medium; Lefrançois et al., 2010) were quantified in aerobic conditions. Samples were diluted 1:100 with a 0.1% peptone solution. This starting dilution was then diluted serially from 1/400 to 1/419,430,400. Wells were incubated at 30°C without light and assessed for visible growth (formation of a bacterial pellet) and/or color change specific to the type of media according to the publication or manufacturer’s protocol after 27 days.
Results

Microcystis responses to Copper Exposures: Cell Density and Chlorophyll a

Following copper exposures, copper concentrations in mesocosms were measured and compared to targeted (nominal) concentrations. Background copper concentrations in untreated controls ranged from 0.016-0.030 mg Cu/L. Measured copper concentrations were 0.38±0.05, 0.71±0.19, and 2.0±0.17 in comparison to targeted concentrations of 0.4, 0.8, and 2.0 mg Cu/L, respectively. In the present study, results are presented and discussed in terms of targeted copper concentrations (0.4, 0.8, and 2.0 mg Cu/L). In mesocosms, initial \textit{M. aeruginosa} cell densities and chlorophyll \textit{a} concentrations ranged from ~9.0x10^5-2.4x10^6 cells/mL and ~1,500-2,400 µg chlorophyll \textit{a}/L, respectively. 5d following exposures to 0.4, 0.8, and 2.0 mg Cu/L, \textit{M. aeruginosa} cell densities decreased 83, 93, and 97%, respectively, in comparison to untreated controls. (Figure 5.1a). Chlorophyll \textit{a} concentrations decreased 74, 94, and 98% compared to untreated controls 5d post exposure to 0.4, 0.8, and 2.0 mg Cu/L, \textit{M. aeruginosa} cell densities decreased 83, 93, and 97%, respectively, in comparison to untreated controls. (Figure 5.1b).

MC-LR Concentrations

\textit{MC-LR}_{Tot} Concentrations

In untreated controls, MC-LR\textsubscript{Tot} concentrations increased at an average rate of 3.6 µg MC-LR/L per day and increased a total of 22±51% from initial MC-LR concentrations over the 6d experimental period (Table 5.3 and 5.4). In copper treatments (0.4, 0.8, and 2.0 mg Cu/L), MC-LR\textsubscript{Tot} concentrations declined 87±5, 97±1 and 97±1%, respectively, over the experimental period (6d). MC-LR\textsubscript{Tot} extents of removal on each
sampling day were differed between treatments (Figure 5.2a, Table 5.3). Degradation rates were significantly different between all copper exposures and untreated controls (based on 95% confidence intervals; Table 5.4). Following exposure to the lowest copper concentration tested (0.4 mg Cu/L), MC-LR_{Tot} concentrations degraded at an average rate of 6.8 MC-LR_{Tot}/L d^{-1} (Table 5.4, Figure 5.2a) with ~87% removal by 120h (6d) post copper exposure (Table 5.4, Figure 5.2a). Following exposure to 0.8 mg Cu/L, MC-LR_{Tot} concentrations degraded at an average rate of 16.6 MC-LR_{Tot}/L d^{-1} (Table 5.4, Figure 5.2a) with >90% removal by 48h (2d) post copper exposure. The highest copper concentration tested (2.0 mg Cu/L) resulted in MC-LR_{Tot} degradation at an average rate of 10.8 MC-LR_{Tot}/L d^{-1} (Table 5.4) with greater than 90% removal by 84h (3.5d) post-copper exposure (Table 5.3, Figure 5.2a).

MC-LR_{Tot} degradation rates were calculated using linear regression for slopes where >87-95% of MC-LR_{Tot} degradation occurred and where the lowest measured MC-LR_{Tot} concentrations plateaued (i.e. no longer decreased with time). Therefore, sampling times (i.e. 12 hour increments) incorporated into calculated rates were different for each treatment. Sampling points between 0-168h, 0-120h, 0-60h, and 0-84h were used to calculate MC-LR_{Tot} degradation rates for untreated controls, 0.4, 0.8, and 2.0 mg Cu/L exposures, respectively.

**MC-LR_{Aq} Concentrations**

Pre-treatment MC-LR_{Aq} concentrations in mesocosms ranged from 0.2-2.0 µg MC-LR/L (i.e. 0.4-4.0% of MC-LR_{Tot}). Within 12-24h post copper exposure, the
percentage of MC-LR present as MC-LR\textsubscript{aq} was between 2±2\%, 8±2-18±14\%, 69±20-120±22\%, and 88±13-78±14\% for untreated controls, 0.4, 0.8, and 2.0 mg Cu/L exposures, respectively (Figure 5.2a and 5.2b). Through the 6d experimental period, MC-LR\textsubscript{aq} concentrations did not exceed 4 µg MC-LR/L in untreated controls. MC-LR\textsubscript{aq} concentrations decreased to pre-treatment concentrations (<4 µg MC-LR/L) by 48-72h (2-3d), 48h (2d), and 72-84h (3-3.5d) post-copper exposure for 0.4, 0.8, and 2.0 mg Cu/L, respectively. MC-LR\textsubscript{aq} concentrations decreased at a rate of 0.1, 1.2, 28.8, and 9.4 µg MC-LR\textsubscript{aq}/L d\textsuperscript{-1} for untreated controls, 0.4, 0.8, and 2.0 mg Cu/L exposures, respectively (Table 5.4). For MC-LR\textsubscript{aq} degradation rates, sampling points between 0-168h, 24-108h, 24-48h, and 12-84h were used to calculate MC-LR\textsubscript{aq} degradation rates for controls, 0.4, 0.8, and 2.0 mg Cu/L exposures, respectively.

**Bacterial Analyses: Density, Diversity, and Relative Abundance**

Density of bacteria was similar between untreated controls and copper exposures of 0.4 mg Cu/L (MPN ~ 10\textsuperscript{9}; Table 5.4). Following exposure to 0.8 and 2.0 mg Cu/L, bacterial density decreased by an order of magnitude to ~ 10\textsuperscript{8} (MPN; Table 5.4). Diversity of total bacteria present in samples was lowest in untreated controls and increased following exposure to 0.4, 0.8, and 2.0 mg Cu/L (Table 5.4). In copper treatments (0.4, 0.8, and 2.0 mg Cu/L), total bacterial diversity was similar (~24-27 [Simpson’s Reciprocal Index]; Table 5.4).

Bacteria identified in samples as potential MC degraders included *Novosphingobium, Rhizobium, Sphingosinicella, Sphingomonas, and Sphingopyxis*. The
most abundant MC-degrading bacteria genera present (in terms of % genera of total bacteria genera present) was *Novosphingobium* (Figure 5.3). Relative abundance of all MC-degrading bacteria present in samples increased with increased copper exposure concentrations, from less than 1% of the total bacteria community in untreated controls and 0.4 mg Cu/L exposures to ~1-3% in 0.8 and 2.0 mg Cu/L exposures (Figure 5.3).
Discussion

Both extent of MC-LR release and subsequent rates of MC-LR decline differed among copper treatments (i.e. copper exposure concentrations). Consistent with other studies, proportion of MC-LRAq was positively related to copper concentration (Tsai 2015, Iwinski et al. 2016a, Iwinski et al. 2016b), where exposure concentrations of 0.4 mg Cu/L resulted in approximately 60% less MC-LRAq than exposures of 0.8 and 2.0 mg Cu/L. However, within the range of copper concentrations tested (i.e. 0.4, 0.8, and 2.0 mg Cu/L), degradation rates of MC-LR were not related to copper concentration (i.e. rates of MC-LR decline did not consistently decrease with increasing copper concentration as hypothesized).

Slowest decline in MC-LR concentrations (except for untreated controls where MC-LRTot concentrations increased over the 6d experimental period), occurred in the lowest tested copper exposures (0.4 mg Cu/L). However, this was likely not due to decreased rates of bacterial degradation, but rather a slower decline in *M. aeruginosa* populations and more gradual release of MC-LR compared to other treatments (0.8 and 2.0 mg Cu/L). MC-LR is not subject to degradation inside *Microcystis* cells, and must be present as MC-LRAq for bacterial degradation to occur. Therefore, as 0.4 mg Cu/L resulted in more gradual release of MC-LRAq over the 6d experimental period, decreases in MC-LRTot concentrations also occurred more gradually. In addition, bacterial density was not significantly different between untreated controls and 0.4 mg Cu/L exposures (MPN >10^9 for 0.4 mg Cu/L exposures and untreated controls).
The slower decline and more gradual release of MC-LR in copper exposures of 0.4 mg Cu/L compared to copper exposures of 0.8 and 2.0 mg Cu/L, may have been due to the multiple mechanisms of action through which copper can elicit toxicity on a cyanobacterial cell (i.e. binding to non-copper specific proteins, disruption of photosynthetic processes, and loss of cell membrane integrity through osmotic imbalance and production of reactive oxygen species; Qian et al. 2010, Jancula and Marsalek 2011, Stevenson et al. 2013, Zhou et al. 2013, Fan et al. 2014). Iwinski et al. 2016b hypothesized that lower copper exposures may result in decreased cellular function and resulting Microcystis population decline, without rapid cell lysis and MC-LR release which can occur at relatively higher copper concentrations.

In copper exposures where rapid (i.e. 12-24h) release of MC-LR to the aqueous phase occurred (i.e. 0.8 and 2.0 mg Cu/L), higher copper concentrations (2.0 mg Cu/L) resulted in significantly lower MC-LR degradation rates (9-10 µg MC-LR/L d⁻¹ compared to 15-28 µg MC-LR/L d⁻¹). In this case, differences in rates of MC-LRₜₒᵗ and MC-LRₐ₉₉ degradation between copper concentrations may have been due changes in bacterial assemblages. Although bacterial diversity and density were not significantly different between 0.8 and 2.0 mg Cu/L exposures, changes in bacterial assemblages did occur as indicated by significant changes in the proportion of MC-degrading bacteria present. Despite significantly different degradation rates following exposures to 0.8 compared to 2.0 mg Cu/L, these rates manifested as only a 1.5d difference in MC-LR removal times (i.e. >90% removal occurred in 48h vs. 84h).
A number of bacteria have been identified as capable of MC degradation. A review of MC-LR degrading bacteria determined that most were primarily Proteobacteria, with several strains from the sphingomonads; other genera included *Methylbaccillus, Paucibacter, Arthrobacter, Bacillus*, and *Lactobacillus* (Dziga et al. 2013, Kormas and Lymperopoulou 2013, Schmidt et al. 2014). In this study, the genera present that have been isolated and identified as potential MC degraders include (based on literature review; Dziga et al. 2013, Kormas and Lymperopoulou 2013, Schmidt et al. 2014) *Novosphingobium, Rhizobium, Sphingosinicella, Sphingomonas*, and *Sphingopyxis*. Increases in the relative abundance of these MC-degrading bacteria with increased copper concentrations may have been due to increased concentrations of MC-LR_{Aq} present following release of MC from *Microcystis*, and/or potentially due to differential sensitivities of MC-degraders compared to other bacteria present (i.e. non-MC degrading bacteria). The increased relative abundance in MC-degrading genera with increased copper concentration is consistent with a previous laboratory study that measured copper influences on bacterial communities (Iwinski et al. in review, 2016).
Conclusions

Results of this study indicate that copper from algaecide applications can influence bacterial assemblages as well as rates of MC-LR degradation and decline. However, these differences were not only dependent on changes in bacterial assemblages, but also responses of the cyanobacteria, *Microcystis*, to copper concentrations. Lower copper exposure concentrations resulted in slower decline of *Microcystis* populations and slower release of MC-LR to the aqueous phase, thereby resulting in more gradual declines in MC-LR concentrations. In relatively higher copper concentrations where MC-LR was rapidly released to the aqueous phase (near and above maximum label concentrations of the tested algaecide; 1.0 mg Cu/L), the highest copper exposure (2.0 mg Cu/L) resulted in slower MC-LR degradation rates than 0.8 mg Cu/L exposures. However, copper exposures 2x the allowable concentration of the tested algaecide (2.0 mg Cu/L) resulted in >97% MC-LR degradation in less than 4d, and concentrations within the range of copper concentrations that can be applied for algaecidal use (0.8 mg Cu/L) resulted in >97% MC-LR degradation in 48h. Based on data from the present study, typical copper algaecide applications (i.e. ≤1.0 mg Cu/L) will likely not result in persistence of MC-LR$_{Tot}$ or MC-LR$_{Aq}$ concentrations. In addition, increases in MC-LR$_{Tot}$ concentrations in untreated controls indicate potential risks of no action decisions for MC-LR producing *M. aeruginosa*. 
References


### Table 5.1 Experimental Conditions

<table>
<thead>
<tr>
<th>Experimental Condition</th>
<th>Field (Mesocosm) Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range of initial cell densities (cells/mL) in mesocosms</td>
<td>~9.0x10^5-2.4x10^6 cells/mL</td>
</tr>
<tr>
<td>Range of initial chlorophyll a (µg/L) in mesocosms</td>
<td>~1,500-2,400</td>
</tr>
<tr>
<td>Cell description/ size (µm)</td>
<td>Colonial/ ~4-5</td>
</tr>
<tr>
<td>Initial MC-LR_{Aq} (µg/L)</td>
<td>0.2-4.0</td>
</tr>
<tr>
<td>Initial MC-LR_{Tot} (µg/L)</td>
<td>29-52</td>
</tr>
<tr>
<td>Average water volume (L) in enclosures</td>
<td>49±7</td>
</tr>
<tr>
<td>pH</td>
<td>9.08</td>
</tr>
<tr>
<td>Dissolved O₂ (mg O₂/L)</td>
<td>&gt;8.0</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO₃/L)</td>
<td>80</td>
</tr>
<tr>
<td>Hardness (mg CaCO₃/L)</td>
<td>56</td>
</tr>
<tr>
<td>Conductivity (µS/cm)</td>
<td>200.8</td>
</tr>
<tr>
<td>Water temperature (°C), average minimum and maximum per day (May 22-26)</td>
<td>~20-30</td>
</tr>
<tr>
<td>Average minimum and maximum light intensity (Lux) ~2cm below water surface (May 22-26)</td>
<td>183-60,622</td>
</tr>
<tr>
<td>Light:dark duration (h) (May 22-26)</td>
<td>~14:10</td>
</tr>
<tr>
<td>P, K, Ca, Mg, Zn, Cu, Mn, Fe,</td>
<td>0.21, 26.7, 14.3, 6.0, &lt;0.01,</td>
</tr>
<tr>
<td>S, B, Na, Cl (mg/L)^*</td>
<td>&lt;0.01, &lt;0.01, 0.06, 4.2, 0.03,</td>
</tr>
<tr>
<td></td>
<td>7.29, 20.8</td>
</tr>
</tbody>
</table>

*Elemental analysis performed by Clemson Agricultural Service Laboratory using inductively coupled plasma (ICP) (APHA 2012)

### Table 5.2 Characteristics of Cutrine-Plus®

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cutrine-Plus®</th>
</tr>
</thead>
</table>

140
<table>
<thead>
<tr>
<th><strong>Active ingredient</strong></th>
<th>Copper</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Active ingredient</td>
<td>9.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Maximum label concentration as copper</strong></td>
<td>1 mg/L&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Formulation</strong></td>
<td>Copper ethanolamine complex&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Appearance</strong></td>
<td>Blue liquid</td>
</tr>
<tr>
<td><strong>Water solubility</strong></td>
<td>Miscible&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Boiling point (°C)</strong></td>
<td>100&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Specific gravity (g/cm³)</strong></td>
<td>1.22</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>10.3-10.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Physical and chemical characteristics are of the original compound unless otherwise noted.

<sup>*</sup> Can mix with water in all proportions.
<sup>a</sup> Applied Biochemists product label
<sup>b</sup> Applied Biochemists MSDS
<sup>c</sup> Murray-Gilde et al. 2002
### Table 5.3 Percent removal of MC-LR$_{Tot}$ post-copper exposure

<table>
<thead>
<tr>
<th>Copper Concentration</th>
<th>12</th>
<th>24</th>
<th>36</th>
<th>48</th>
<th>60</th>
<th>72</th>
<th>84</th>
<th>96</th>
<th>108</th>
<th>120</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-5±14</td>
<td>-5±25</td>
<td>-21±22</td>
<td>6±45</td>
<td>6±42</td>
<td>31±43</td>
<td>18±40</td>
<td>28±34</td>
<td>35±30</td>
<td>22±51</td>
</tr>
<tr>
<td>0.4 mg Cu/L</td>
<td>-2±12</td>
<td>-1±19</td>
<td>-35±14</td>
<td>-37±17</td>
<td>-20±25</td>
<td>-32±13</td>
<td>-55±9</td>
<td>-76±22</td>
<td>-87±12</td>
<td>-87±5</td>
</tr>
<tr>
<td>0.8 mg Cu/L</td>
<td>-23±16</td>
<td>-36±24</td>
<td>-48±6</td>
<td>-92±2</td>
<td>-94±3</td>
<td>-95±1</td>
<td>-94±2</td>
<td>-94±2</td>
<td>-96±1</td>
<td>-97±1</td>
</tr>
<tr>
<td>2.0 mg Cu/L</td>
<td>-21±15</td>
<td>-24±24</td>
<td>-31±25</td>
<td>-56±19</td>
<td>-78±10</td>
<td>-86±6</td>
<td>-91±5</td>
<td>-95±2</td>
<td>-96±2</td>
<td>-97±1</td>
</tr>
</tbody>
</table>

Average percent removal of MC-LR$_{Tot}$ ± standard deviation, post copper exposure at each sampling time. A negative sign indicates an average decrease in MC-LR$_{Tot}$ concentrations. HAT=hours after copper treatment.
Table 5.4 Rates (k) and $R^2$ for MC-LR$_{Tot}$ degradation; bacterial diversity (Simpson’s reciprocal) and bacterial density (MPN) following copper exposures

<table>
<thead>
<tr>
<th>Copper (mg/L)</th>
<th>k (range)</th>
<th>$R^2$ (range)</th>
<th>k (range)</th>
<th>$R^2$ (range)</th>
<th>Simpson’s Reciprocal Diversity Index (range)</th>
<th>Bacteria Density (MPN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.6 (0.5, 6.2)</td>
<td>0.01-0.89</td>
<td>-0.10 (-0.2, 0.05)</td>
<td>0.14-0.70</td>
<td>7 (5-8)</td>
<td>&gt;1x10^9</td>
</tr>
<tr>
<td>0.4</td>
<td>-6.8 (-8.0, -4.1)</td>
<td>0.77-0.89</td>
<td>-1.2 (-1.9, -0.2)</td>
<td>&lt;0.001-0.81</td>
<td>27 (20-32)</td>
<td>&gt;1x10^9</td>
</tr>
<tr>
<td>0.8</td>
<td>-16.6 (-18.6, -15.1)</td>
<td>0.84-0.95</td>
<td>-28.8 (-34.4, -24.2)</td>
<td>0.97-0.99</td>
<td>24 (15-30)</td>
<td>~1x10^8</td>
</tr>
<tr>
<td>2.0</td>
<td>-10.8 (-10.0, -11.8)</td>
<td>0.89-0.93</td>
<td>-9.4 (-11.3, -7.5)</td>
<td>0.87-0.95</td>
<td>24 (16-30)</td>
<td>~1x10^8</td>
</tr>
</tbody>
</table>

$R^2$ and k ranges represent the range of calculated $R^2$ and k values measured for treatment replicates (i.e. each mesocosm) within each copper exposure concentration (i.e. control, 0.4, 0.8, and 2.0 mg Cu/L exposures). Bacterial diversity and density calculated from samples collected 72 hours post copper exposure.
Figure 5.1 Cell density (a) and chlorophyll $a$ concentrations (b) 5 days following exposures to copper as Cutrine-Plus®
Figure 5.2 Total (a) and aqueous (b) MC-LR concentrations measured over time following exposure to copper at 0, 0.4, 0.8, and 2.0 mg Cu/L as Cutrine-Plus®
Figure 5.3 Relative abundance of MC-LR degrading bacteria 72 hours following exposure to Cutrine-Plus®
CHAPTER SIX
CONCLUSIONS

Microcystins (MCs) are algal produced toxins that pose human and ecological health risks in freshwater resources. Use of algaecides to manage MC-producing cyanobacteria in aquatic systems is influenced by studies stating that copper-algaecide applications result in release of MCs from cyanobacteria and subsequent persistence of MCs due to effects on microcystin-degrading bacteria. The currently accepted model hypothesizes that copper-algaecides will result in MC release and persistence regardless of copper form and concentration, and consequently may increase risks of MCs in aquatic systems. This research tested hypotheses of whether these factors (copper form and concentration) as well as consequences of “no-action” decisions (i.e. allowing cyanobacteria and microcystin concentrations to remain untreated) would alter this currently accepted model.

The first study, *Cellular and Aqueous Microcystin-LR Following Laboratory Exposures of Microcystis aeruginosa to Copper Algaecides*, was a laboratory study using cultured *M. aeruginosa*, formulated water, and two forms of copper-algaecides. The study measured the relationship between copper concentration and MC release, compared copper formulations (in terms of MC release and total MC concentrations), and compared total MC concentrations in untreated controls to copper treatments over time. The results of the study emphasized that copper concentration is an important factor in responses of *M. aeruginosa* in terms of MC-LR release. The positive relationship between copper
concentration and aqueous MC-LR at effective algaecide concentrations (0.07-1.0 mg Cu/L) demonstrated that lower copper concentrations were as effective as higher concentrations in controlling *M. aeruginosa* while decreasing the total amount of MC-LR (compared to the control), and minimizing the proportion of MC-LR released from the cellular to aqueous phase. Results of the study indicated that for the specific algae and formulated water, copper formulation did not significantly alter *M. aeruginosa* responses in terms of MC release. Finally, MC-LR$_{\text{Tot}}$ concentrations in control samples continued to increase over time, implicating potential consequences of “no-action” decisions in terms of MC-LR$_{\text{Tot}}$.

The study, *Comparison of Laboratory and Field Responses of the Microcystin Producing Cyanobacteria (Microcystis sp.) to Copper-Based Algaecide Exposures*, measured relationships between copper concentration and MC release over time in laboratory experiments with cultured *M. aeruginosa* in formulated BG-11 medium, laboratory experiments with field collected *M. aeruginosa* and water, and in field mesocosms with *M. aeruginosa*. A positive correlation was measured between MC-LR release (MC-LR$_{\text{Aq}}$) and copper concentrations in both laboratory and field studies. The incremental release of MC-LR from *M. aeruginosa* within a range of effective copper concentrations at which cell density and chlorophyll *a* responses remained constant demonstrates that lower copper concentrations were as effective as higher concentrations in controlling *M. aeruginosa* while decreasing the amount of MC-LR released to aqueous phase. Although laboratory cultures of *M. aeruginosa* in BG-11 medium were more
sensitive to copper exposures than field algae in site water, laboratory experiments with field collected algae produced similar results to SFSs with the same algae and water. This emphasizes the utility of preliminary laboratory experiments for predicting \textit{M. aeruginosa} responses in the field. The results also highlight that using copper more efficiently (i.e. utilizing the minimum concentration needed for control of the target algae) decreased not only the amount of copper-algaecide required, but can also minimize MC-LR release.

The laboratory study, \textit{Influence of Copper Algaecide Concentration and Formulation on Microcystin-LR Degradation}, tested the effects of copper-algaecide concentrations and formulations on MC-LR degradation and bacterial communities. Results indicated that copper concentrations can influence degradation rates of MC-LR following copper-algaecide exposures. However, exposures to copper concentrations registered for use (i.e. 0.1-1.0 mg Cu/L as Cutrine-Plus® and Algimycin-PWF®) did not influence MC-LR$_{Tot}$ degradation compared to untreated controls. Relative abundance of MC-LR-degrading bacteria increased following exposure to increasing copper concentrations. This suggests that MC-LR degrading bacteria may be less sensitive than other genera to copper algaecides. Copper formulation did not significantly alter degradation rates or bacterial composition at comparable copper exposures.

The study, \textit{Microcystin-LR Release and Degradation Following Exposures of M. aeruginosa to a Chelated Copper Algaecide}, measured MC-LR release and subsequent rates and extent of MC-LR degradation following exposure to different concentrations of
a copper-algaecide. Results indicated that copper from algaecide applications can influence bacterial assemblages as well as rates of MC-LR degradation and decline. However, these differences were dependent not only on changes in bacterial assemblages, but also responses of the cyanobacteria, *M. aeruginosa* to copper concentrations. Lower copper exposure concentrations resulted in slower decline of *M. aeruginosa* populations and slower release of MC-LR to the aqueous phase, thereby resulting in more gradual declines in MC-LR concentrations. In relatively higher copper concentrations where MC-LR was released rapidly to the aqueous phase (near and above maximum label concentrations of the tested algaecide; 1.0 mg Cu/L), the highest copper exposure (2.0 mg Cu/L) resulted in slower MC-LR degradation rates. However, copper exposures near (0.8 mg Cu/L) and above (2.0 mg Cu/L) allowable copper-algaecide concentrations resulted in MC-LR degradation in less than 48h and 96h, respectively. Based on data from the present study, typical copper algaecide applications (i.e. ≤1.0 mg Cu/L) will likely not result in persistence of MC-LR\textsubscript{Tot} or MC-LR\textsubscript{Aq} concentrations.

The research presented in this dissertation emphasizes that copper concentration is an important factor in extent of MC release from *Microcystis* cells, and in rates and extents of MC-LR degradation. The data provide context for the previously accepted model (Figure 1.1) regarding MC release and persistence following copper exposures. Results of these studies indicate that although the previously accepted model may be partly accurate, it is likely predictive of relatively higher copper exposure concentrations above what is typically applied as algaecides (Figure 6.1). Relatively lower copper
exposure concentrations within typical applications (i.e. ≤1.0 mg Cu/L) resulted in less MC release and did not cause significant alterations in MC-LR degradation.

Figure 6.1 Previous model compared to model derived from current research (new model) illustrating effects of copper concentration on MC release from Microcystis and subsequent persistence as a result of bacterial degradation
Overall, these data provide a more thorough understanding of the influences of copper algacide concentration and formulation on MC-LR release, MC-LR degradation, and potential consequences of “no-action” management decisions. These studies can be used to inform more accurate risk evaluations and use of copper-based algacides for management of MC-LR producing *M. aeruginosa.*