Monitoring, quantifying, and controlling the effects of cyanobacterial blooms and toxins

by

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Abstract

Cyanobacterial blooms and toxins have harmful impacts on global aquatic ecosystems. Within this, their impact on the water quality of freshwater systems are particularly troublesome. My research investigated methods to both monitor and control cyanobacterial blooms and toxins to assist resource managers with this pressing aquatic resource issue.

My monitoring research included identifying the environmental drivers of microcystin, a hepatotoxin produced by select cyanobacteria, using both a linear meta-analytical analysis and non-linear generalized additive modeling approach. Linear meta-analysis of 2,643 global fresh waterbodies indicated that chlorophyll, total dissolved phosphorus, total phosphorus, and Secchi disk depth were the strongest water quality parameters related to microcystin occurrence. On the other hand, non-linear analysis of 2,040 global fresh waterbodies indicated that total nitrogen, turbidity, pH, and Secchi disk depth were the strongest water quality parameters related to microcystin occurrence. Although similarities were found between these two approaches, my findings suggest trends in cyanobacterial data may be non-linear, and utilizing non-linear analyses to assess such data are recommended.

To control cyanobacterial blooms, I compared the effectiveness of 7 different algaecides (including copper-, hydrogen peroxide- (H_2O_2), peracetic acid-, and clay-based products) in a 35-day field experiment, and also assessed the nuanced effectiveness of H_2O_2 under varying environmental conditions. In general, it was found that copper-based products remain the most efficient and cheapest choice to reduce total phytoplankton biomass in aquaculture systems. However, peracetic acid-based products effectively reduced cyanobacteria while having marginal effects on beneficial algae and zooplankton. Such algaecides could be effective alternatives to copper-based products depending on the outcomes sought by resource managers.

Lastly, my laboratory study of H_2O_2 assessed its effectiveness at reducing cyanobacteria under different environmental conditions, including varying dissolved organic matter concentrations, temperatures, and starting phytoplankton concentrations. Neither variation in dissolved organic matter concentration nor temperature influenced the effectiveness of H_2O_2 at reducing cyanobacteria. However, initial phytoplankton density as well as H_2O_2 dose greatly influenced the effectiveness of the algaecide. Thus, water resource managers are encouraged to consider how ambient conditions may alter the ability H_2O_2 to control algal blooms prior to treatment.

Acknowledgments

I dedicate this dissertation to my family and fiancé, Samantha. Their support has brought me to this moment. Thank you to Dr. Wilson, my committee members, and the other members of the Wilson lab for their assistance in this research.

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Chapter 1

Identifying the drivers of microcystin in freshwater lakes for applied resource management: a global meta-analysis

Abstract

Cyanobacterial blooms can have negative effects in freshwater ecosystems, including the production of toxic secondary metabolites that threaten aquatic and terrestrial organisms. Of the many known cyanobacterial toxins, microcystin (MC) has been widely studied and found in diverse systems. Despite this, prior studies have provided little quantitative consensus regarding the typical conditions likely to influence MC occurrence unlike cyanobacterial biomass. This has left uncertainty for resource managers who desire to know the optimal water quality parameters to monitor in systems at risk of cyanotoxin-related issues. The objective of this research is to provide a starting point for resource managers desiring to monitor for the drivers of MC by quantitatively identifying the environmental parameters associated with MC concentrations using monitoring data from lakes and reservoirs over a wide spatial and temporal scale. Environmental (i.e., physical, chemical, and biological) and MC data were retrieved from 131 global sources and quantitatively analyzed using meta-analysis. Correlation coefficients were transformed to a Pearson's correlation coefficient, when needed, and then converted to a Fisher's z-score prior to meta-analysis. The overall dataset included 1,029 effect sizes produced from an estimated 2,643 waterbodies. Commonly measured water quality parameters such as chlorophyll, temperature, and pH were positively correlated with MC, while transparency (Secchi depth) was negatively correlated with MC. Interestingly, 12 of the 15 studied nitrogen parameters, including total nitrogen, were not significantly correlated with MC. In contrast, three of the four studied phosphorus parameters, including total phosphorus, were positively related to MC. Results from

this global synthesis of water quality data quantitatively reinforce the usefulness of commonly measured environmental parameters to monitor for conditions related to MC occurrence.

Introduction

Cyanobacterial blooms can have harmful impacts on global freshwater ecosystems by causing drastic changes in physicochemical conditions (e.g., hypoxia, decreased light penetration, elevated pH; Paerl et al., 2001; Paerl and Otten, 2013) and by the production of secondary metabolites that can be toxic to aquatic organisms, livestock, and humans (Paerl et al., 2001; Graham et al., 2004; Malbrouck and Kestemont, 2006; Rinta-Kanto et al., 2009). Of these cyanobacterial toxins, microcystin (MC; produced by several cyanobacterial genera, including *Microcystis*, *Nostoc*, *Oscillatoria*/*Planktothrix*, *Dolichospermum/Anabaena*, and *Anabaenopsis* [Martins and Vasconcelos, 2009]) is commonly observed in freshwater systems with over 246 structural variants documented (Meriluoto et al., 2017). The ubiquity of MC has prompted many researchers to study the environmental conditions that contribute to its occurrence (Rinta-Kanto et al., 2009; Graham et al., 2017). Field observations have begun to provide a framework of the typical conditions required for a toxigenic cyanobacterial bloom to occur; however, observations across systems and geographical regions indicate variability in the documented conditions mostly likely to influence MC occurrence (Kotak et al. 2000; Billam et al. 2006; Wu et al. 2006; Duong et al. 2012; Gonzalez-Piana et al. 2017). These reports can include observations from only a single waterbody sampled during a single growing season (Wilhelm et al., 2011), and relatively few studies have provided findings generated over multiple years and ecosystems (but see Graham et al., 2004; EPA, 2012; Beaver et al., 2014). These narrow sampling practices have likely contributed to the variability findings across systems regarding MC occurrence. Given our limited understanding of the function of MC, it

has been difficult to generalize the specific conditions most influential to the occurrence of MC despite the wealth of literature published on this topic.

Contributing to the discrepancies in documented findings of the environmental parameters likely to influence MC occurrence is the natural variability of toxin production and the highly dynamic nature of phytoplankton blooms. This includes variation in the relative dominance of toxigenic cyanobacteria in a bloom and the abundance and diversity of cyanobacteria over space and time (Wu et al., 2008; Rinta-Kanto et al., 2009; Li et al., 2017). The ecological function of cyanotoxins is also debated with their generation thought perform as iron scavengers, allelopathic chemicals, or defenses against grazing (among others); suggesting may possible reasons as to when toxins will be present in a freshwater system (Holland and Kinner, 2013). In addition, each freshwater system and respective bloom will be subjected to different physical, chemical, and anthropogenic influences (Rinta-Kanto et al., 2009). These factors increase the difficulty of determining the primary conditions likely to produce a cyanobacterial bloom, particularly across large geographical regions. Yet, determining these trends is needed given that cyanobacterial blooms are a global issue, the occurrence of which is expected to increase in frequency and severity through both anthropogenic-promoted eutrophication and climate change (Paerl and Paul, 2012). Considering the ubiquity of toxigenic cyanobacterial taxa and their relatively wide range of environmental tolerances (Paerl et al., 2001), it is likely that the environmental conditions contributing to MC occurrence in one geographical region will also contribute to its occurrence in other regions.

The negative health effects of microcystins and other cyanobacterial toxins threaten drinking water (WHO, 2020) and recreational areas (Francy et al., 2016; Francy et al., 2020).

Testing for MC does exist, where municipalities determine when their water system has the toxin present. However, quantitative testing can be costly and requires specialized training and analytical infrastructure (Yinxia et al., 2017). This form of monitoring also only indicates when the toxin is present and does not assist in preventing its occurrence or in determining why it occurred. Understanding the trends between environmental conditions and MC occurrence would assist resource managers in monitoring and mitigating these conditions as they strive to reduce the costs and health risks associated with MC.

Bearing in mind the need for a greater understanding of the factors that mediate MC occurrence, the objective of this research was to assess the strength of the relationship between various environmental parameters and MC occurrence in freshwater ecosystems using meta-analysis techniques and incorporating as much pertinent literature available. These findings will provide resource managers with a cursory, but targeted set of parameters to monitor as they begin to determine MC occurrence in waterbodies important to human health and industry as well as to help delineate the main drivers of eutrophication as it relates to cyanobacterial blooms and their associated toxins. Moreover, results will provide a quantitative assessment as to whether the most commonly collected water quality parameters are most associated with MC occurrence.

Methods

Data collection

Three primary sources were used to acquire data, including published journal articles, the National Water Information System database of the U.S. Geological Survey (USGS, 2018), and the 2007 and 2012 National Lake Assessments conducted by the U.S. Environmental Protection Agency (NLA; EPA, 2007 and 2012). Data acquisition from each source is described separately

in the following paragraphs.

Published articles were retrieved using the Web of Science database. A literature search was performed in February 2018 by combining each of the keywords “lake”, “reservoir”, “environment”, “parameters”, “nutrients”, “variables”, “environmental parameters”, and “environmental variables” with “microcystin”; the search returned 3,332 articles. Studies were included in the meta-analysis if they (1) were observational field studies (i.e., not experimental in nature), (2) were from a freshwater reservoir or lake, defined as a system with little-to-no-flow (i.e., no unimpeded rivers or streams), (3) provided data for ≥5 samples collected at any given site, and (4) provided numerical sampling data in figures, tables, or supplementary files. Seventy-nine studies met these criteria (see Appendix A).

The National Water Information System of the U.S. Geological Survey was accessed in February 2018 to obtain real-time field sampling data containing MC values and associated environmental parameters from sites within the United States (USGS, 2018). Data were used if they followed the four guidelines; fifty sites were chosen. Many sites contained a wide array of measured parameters (e.g., heavy metals, organic and inorganic chemicals, physical measurements). Parameters that were not reported in the obtained published studies were largely removed. Lastly, common symbols were reported with published values throughout the USGS dataset were assigned half values or excluded from the dataset (Appendix Table A1).

Data were also available from an estimated 1,161 and 1,137 lakes or reservoirs as part of the recent EPA NLA in 2007 or 2012, respectively, which included MC data (EPA, 2007; 2012). Of these waterbodies, 460 of the 2007 dataset were estimated to be resampled in 2012, resulting in 701 newly sampled lakes or reservoirs in the 2012 dataset. Sites were typically sampled one or two times. If it was observed that a study used data that were also provided in the NLA or USGS

datasets (e.g., Beaulieu et al., 2013; Beaver et al., 2014; Harris and Graham, 2017), the study was discarded and only the original raw data were used. Despite their size, the 2007 and 2012 NLA datasets were assigned one correlation per environmental parameter for use in this meta-analysis.

From the three data sources, a total of 131 articles and datasets that contained physicochemical (i.e., temperature, dissolved oxygen, pH, Secchi depth [transparency], conductivity), chemical (i.e., nitrogen, nitrate, nitrite, ammonia, phosphorus, phosphate), algal biomass (total chlorophyll and chlorophyll-a), and cyanotoxin (microcystin) data, independent of time or season were included. Phosphate included both phosphate and orthophosphate. Total microcystin measurements were used if available (23.7% of studies), but intracellular (particulate) microcystin (26.7%), microcystin + nodularin (USGS, 2018; 38.9%), and the microcystin variant, microcystin-LR (10.7%), were all also used as alternatives if no other options were available. Analytical methods to obtain MC included enzyme-linked immunosorbent assay, high performance liquid chromatography, liquid chromatography coupled with mass spectrometry, and protein phosphatase inhibition assay.

Correlation coefficient development

Commonly measured correlations of the relationship between a specific environmental parameter and microcystin concentration served as the foundation for this synthesis. From the published articles, three correlation coefficients provided by the authors were primarily used, including Pearson's *r*, Spearman's *rho*, or Kendall's *tau*. Reported correlation coefficients that were not otherwise specified were treated as Pearson's. It was observed that 68% of the peer reviewed publications used in this analysis reported environmental parameters to MC occurrence specifically using some form of correlation coefficient. In some cases, Pearson correlation coefficients were also generated for studies that did not directly provide this value. In these

instances, data were extracted from a figure using the *metaDigitize* package in R. The *cor.test* package in R was then used to generate Pearson correlation coefficients from the obtained data. Lastly, Spearman's *rho* or Kendall's *tau* correlation coefficients were transformed to Pearson's *r* using the following two equations:

Kendall's *tau* to Pearson *r*, reported by Rupinski and Dunlap (1996):

$$r = \sin(t * \frac{\pi}{2})$$

where *r* = Pearson's coefficient and t = Kendall's *tau*.

Spearman's *rho* to Pearson *r*, reported by Rupinski and Dunlap (1996):

$$r = 2 * \sin(r_s \frac{\pi}{6})$$

where *r* = Pearson's coefficient and r_s = Spearman's *rho*.

Statistical analyses

Fisher's z (*z*) scores were used as a measure of effect size due to its improved statistical properties (Jiang et al., 2009; Weaver et al., 2018; Ortega et al., 2020). Prior to statistical analyses, the Pearson correlations (*r*) for each environmental variable were converted to Fisher's z-scores using the following equation (Fisher, 1958) in the *metafor* package in R to normalize the distribution of the data (Viechtbauer, 2010):

$$z = 0.5\ x\ ln\left(\frac{1+r}{1-r}\right)$$

Effect sizes (hereby termed 'correlations') were then generated from the Fisher's z-scores using the Robust Multi-Array Average function including the restricted maximum likelihood method found within the *metafor* package. The *metafor* package also produced a weighted variance and standard error for each Fisher's z-score. In addition, a test of

heterogeneity (I^2) and 95% confidence intervals were generated for each environmental parameter correlated with MC, separately. Lastly, a visual test for publication bias was generated via a funnel plot depicting effect size, as Fisher's z, against standard error using the *metafor* package (Appendix Figure A1). Funnel plots depict heterogeneity of study estimates, and values that fall within the 95% confidence region (triangular region) are considered be free of publication bias (Higgans and Green, 2008), and it was determined that the majority of values used in this analysis fell within this region.

To ease the interpretation of the study results, a Fisher's z-score was converted back to a Pearson correlation coefficient using the equation:

$$r = tanh(Z_r)$$

Where Z_r = Fisher's z-score (Gilpin, 1993).

Pearson correlation coefficients range from -1 (perfect negative correlation) to +1 (perfect positive correlation). Ninety-five percent confidence intervals were used to gauge significance ($p \leq 0.05$). The amount of studies (*n*) used to generate effect size estimates were also reported.

Results

The final dataset was reduced to correlations of 35 environmental parameters with MC totaling 1,029 individual effect sizes across an estimated 2,643 unique waterbodies (Figure 1.1). Most data were collected in the United States, Europe, and China (Figure 1.1). Additional studies were identified in other parts of globe during the literature review but contained data that were not in a usable form and/or did not meet our inclusion requirements. Correlations included a broad range of studies (3-106; Figures 1.2 and 1.3; Appendix Tables A2 and A3) and averaged 36 studies. Heterogeneity also varied widely across environmental parameters (0 - 98%; Figures

1.2 and 1.3). Although most correlations had high heterogeneity (I^2 >50%), I^2 for eight correlations were low (≤ 25%), indicating that either the between-study estimates were extremely homogenous or small sample size precluded accurately estimating among-study variation.

Physical parameters

A statistically significant ($p \leq 0.05$) positive correlation was found between MC and temperature (r = 0.20, n = 106) (Figure 1.2; Appendix Table A2). Secchi depth (measurement of transparency) was the only physical parameter that had a significant negative correlation with MC (r = -0.20, n = 44). The three other physical parameters, turbidity (r = 0.18, n = 34), total suspended solids (r = 0.08, n = 21), and conductivity (r = 0.07, n = 69), assessed in this meta-analysis were not significantly correlated with MC.

Biological and chemical parameters

Chlorophyll (r = 0.45, n = 76), total dissolved phosphorus (r = 0.30, n = 13), total phosphorus (e.g., measure of all phosphorus in a whole water sample) (r = 0.22, n = 92), phosphate (r =0.14, n = 59; p = 0.055), dissolved organic carbon (DOC; r = 0.07, n = 17), nitrate (r = -0.12, n = 74), dissolved nitrogen (r = -0.19, n = 5), and pH (r = 0.12, n = 92) were found to be significant ($p \leq 0.05$) and positively correlated with MC (Figures 1.3 and 1.4; Appendix Tables A3 and A4). Chlorophyll had the largest positive correlation with MC of any parameter assessed in this synthesis (n =76). Of the fifteen nitrogen water quality parameters, correlations for only two parameters were statistically significant and both were negatively correlated with MC (dissolved nitrogen, nitrate; Figure 1.4). The number of studies included for specific nitrogen parameters varied widely (n range = 5 – 82; Appendix Table A4) as did between-study heterogeneity (I^2 = 0 - 98%). Total nitrogen displayed a nearly-zero, non-significant correlation despite having data from a large number of studies included in its effect size (n = 82). Ammonia,

and its derivatives, showed mostly positive, albeit statistically insignificant, correlations with MC (Figure 1.4).

Interestingly, and in contrast to the nitrogenous parameters, all four reported phosphorus parameters displayed positive correlations across a range of studies (n = 13 – 92; Appendix Table A4). Total phosphorus had one of the largest number of studies incorporated into its estimate and displayed a statistically significant positive correlation (r = 0.22; n = 92; $p \leq 0.05$; Figure 1.3). Total dissolved phosphorus (r = 0.3; n = 13) and phosphate (r = 0.14; n = 59; p = 0.55) were also positively correlated with MC (Figure 1.4). Soluble reactive phosphorus was the only phosphorus measure not statistically related to MC.

Discussion

With this meta-analysis, we synthesized global water quality data from both single- and multi-lake surveys to identify the environmental parameters most significantly correlated with MC. A number of large, multi-lake surveys have occurred in recent decades, including those conducted in Canada (Kotak et al., 2000; Giani et al., 2005), the United States (Graham et al., 2004; NLA, 2007, 2013), China (Wu et al., 2006), Europe (Mantzouki et al., 2018), and the Czech Republic (Jančula et al., 2014). However, no survey has assessed the strength of this number of environmental parameters in relationship to MC. Although data from an estimated 2,643 waterbodies were used for this meta-analysis, data largely originated from sources in the northern hemisphere, despite attempts to secure a globally representative dataset during the literature review. Continued availability of open-sourced data and further assessments of lakes in the southern hemisphere will assist in addressing global questions such as those asked in this meta-analysis.

Despite the wealth of monitoring data for freshwater lakes and reservoirs from around the world (Kotak et al., 2000; EPA-NLA 2007, 2012; Wu et al., 2006), relatively few environmental parameters (29%; 10 of 35; including temperature, pH, Secchi disk depth, chlorophyll, DOC, total phosphorus, total dissolved phosphorus, phosphate, nitrate, and dissolved nitrogen) were significantly correlated with MC in this study (Figure 1.1). The limited significance of these variables are not surprising given that the function of most cyanobacterial secondary metabolites, including MC, as well as the factors responsible for their production are poorly understood (Holland and Kinnear, 2013; Paerl and Otten, 2013; Sivonen, 2009). We do know that toxigenic cyanobacterial taxa must be present for toxins to be produced, but the presence of these taxa alone is no guarantee for toxin production, given that cyanobacterial strains vary in their toxin production during population growth and collapse or when exposed to varying environmental conditions (Wiedner et al., 2003; Lyck, 2004; Horst et al., 2014). Additionally, toxin production can vary strongly across different genotypes (Watanabe et al., 1989; Lyck, 2004; Wilson et al., 2006; Yinxia et al., 2017). Our meta-analysis determined those environmental factors that most significantly correlated with MC that water resource managers might consider using when monitoring blooms of MC-producing cyanobacteria. Given that these parameters were present in data collected for this meta-analysis, it is indicative of their global popularity as auxiliary measurements collected to monitor water quality as well as MC occurrences in freshwater systems. However, previously stated, the debate as to what group of parameters are the best to be incorporated into monitoring programs focused on cyanotoxin occurrence remains (Neilan et al., 2013). The parameters found to be significant in this analysis serve as a quantitate indicator to resource managers seeking to better understand the drivers of MC in freshwater bodies that routine limnological variables are still the best option. Significant parameters will be described in

the following paragraphs in which examples will be given as to how parameters are connected with toxigenic cyanobacteria as well as to MC itself. It is important to restate that this analysis measured correlations between environmental parameters and MC specifically. Examples relating environmental parameter effects on toxigenic cyanobacteria are meant to provide a better understanding regarding how a parameter may influence toxic cyanobacteria and therefore the occurrence of MC.

Physical parameters

Temperature

In general, warmer temperatures lead to increased cyanobacterial growth (Paerl and Huisman, 2008), often due to the ability of cyanobacteria to outcompete other phytoplankton genera via unique adaptations (e.g., thermal tolerances, gas vesicles; Paerl and Otten, 2013). Temperature has also been thought to increase toxin production, but findings also state lower temperatures may be more beneficial (Griffith and Gobler, 2020). The findings of our meta-analysis reflect that MC may be attributed to higher temperatures. Numerous optimal temperature ranges for toxigenic cyanobacteria have been reported, with temperatures greater than 20°C generally supporting the formation of toxic blooms (15-20 °C, Billam et al., 2006; ~23 °C, Li et al., 2007; 18-35 °C, Gagala et al., 2012; >23 °C, Rigosi et al., 2015; >25 °C Boutte et al., 2008). Interestingly, studies have also shown that increased temperatures cause an upregulation of the *mcyB* gene (a gene that is part of a group of genes that regulates MC production) in *M. aeruginosa*, with peak upregulation occurring at 25°C (Kim et al., 2005; Scherer et al., 2016). It is suggested that microcystin and other cyanobacterial toxins serve as radical scavengers in cells seeking to limit oxidative stress, and temperature in relation to

increased solar irradiance is likely to increase the occurrence of cyanobacterial toxins (Dziallas and Grossart, 2011).

Secchi depth

Transparency (measured as Secchi depth) displayed a significant negative correlation with MC occurrence. As Secchi depth can be measured with relative ease, we recommend that it be incorporated into water resource monitoring programs, including community-based science programs. Some training is required with this measurement, as it also offers information unrelated to cyanobacterial blooms. For instance, high levels of turbidity, algal cells (both non-toxic cyanobacterial strains and other phytoplankton species), dissolved organic matter, or suspended solids may each cause decreases in Secchi depth, but can equate to very different ecological stressors or processes in freshwater systems (Swift et al., 2006). Despite this, it has been suggested that certain cyanobacterial species are capable of excelling in turbid environments, as select species are, for example, adapted to low-light situations (e.g., *C. raciborskii*) or can remain on the surface of water using gas vesicles to maintain buoyancy circumventing these conditions (e.g., *Microcystis*; Paerl and Huisman, 2008). High turbidity measurements are also correlated to cyanobacterial blooms as higher cell densities increase turbidity measurements (Mihaljević and Stević, 2011), although turbidity was not considered significant in this meta-analysis.

Biological and chemical parameters

Chlorophyll

Eight of the twenty-nine biological and chemical parameters measured in this analysis were statistically significant, including chlorophyll, DOC, total dissolved phosphorus, total phosphorus, phosphate, nitrate, dissolved nitrogen, and pH. Of these significant parameters,

and in the overall analysis, chlorophyll had the greatest correlation slope to MC and is as such recommended to be measured in systems experiencing the threat of MC occurrence. Other algal pigments, such as phycocyanin, could also be measured to cyanobacterial density and may provide a stronger relationship with cyanobacteria (McQuaid et al., 2011; Kasinak et al., 2015), however these measurements are collected less often by water resource managers globally. It should be noted that although chlorophyll and other algal pigments are a beneficial measurement of phytoplankton abundance because of its ease of use, these values alone do not infer that MC will be produced as not all blooms will possess cyanobacterial species capable of producing cyanobacterial toxins, and not all cyanobacterial species will possess the genes required for toxin production even if toxigenic cyanobacterial species dominate (Rinta-Kanto et al., 2009). For instance, Wilhelm et al. (2011), who studied Lake Taihu, China, found the presence of the *mcyA* genes needed for MC production in only three of the ten sites sampled despite all sites having DNA from *Microcystis* present. Regardless of these considerations, measurement of cyanobacterial pigments, such as chlorophyll and/or phycocyanin, represent some of the most effective methods to quickly and efficiently monitor the progression of an algal or cyanobacterial bloom.

Dissolved organic carbon

Dissolved organic carbon displayed the weakest slope, albeit still statistically significant correlation with MC. As observed with other environmental parameters, the mechanism through which DOC is important to MC production and cyanobacterial growth is questioned within the literature. The correlation occurs between cell density and DOC occurs because dissolved organic matter is produced as a by-product of cyanobacterial cells (Tessarolli et al., 2018) or matter is released during cyanobacterial cell lysis (Paerl et al., 2001). Therefore, the

relationship between DOC and MC occurrence may exist because DOC is a product of the cyanobacterial bloom itself. Others suggest that DOC is important to cyanobacteria because it is used for growth (Pearl et al., 2001). Specifically, it is thought that the DOC is either assimilated directly by cyanobacteria or by the bacterial matrix surrounding or residing with the mucilage or cyanobacterial cells, thus increasing cell abundance (Paerl et al., 2001; Znachor and Nedoma, 2010; Hooker et al., 2019). Further delineation on whether the correlation observed between DOC and MC occurrence is attributed to DOC fueling cyanobacterial abundances or if DOC is a by-product of established blooms is needed or perhaps is a combination of the two factors. Carbon sources may also be terrestrial sources and this should also be considered.

Total phosphorus, total dissolved phosphorus, phosphate, nitrate, and dissolved nitrogen.

Total phosphorus and the other phosphorus parameters in this meta-analysis had positive correlations with MC that were typically stronger than that of nitrogenous parameters, which yielded largely weak-to-negative correlations. These findings may suggest that phosphorus contributes more to MC occurrence globally than other nutrients and resource managers experiencing MC issues should consider limiting its presence and/or entrance into a system. The findings of this meta-analysis reflect the conclusions of previous studies that reported phosphorus had a larger importance to eutrophication and algal/cyanobacterial biomass versus nitrogen or nutrient ratios (i.e., C:N:P Schindler et al., 1974 and Schindler et al., 2008; N to P Kotak et al. 2000), and that cyanobacteria often experience phosphorus limitation in freshwater systems (Dignum et al., 2005). Also, it has been suggested that non-toxic *Microcystis* strains outcompete toxic strains in low nutrient conditions, but toxic strains dominate at higher nitrogen and phosphorus concentrations (Vezie et al., 2002). Moreover, *M. aeruginosa* grown in phosphorus-limited conditions produced MC with the addition of phosphorus (Oh et al., 2000).

To minimize the importance of nitrogen to MC occurrence is not the objective of this study. Numerous surveys have shown that nitrogen is correlated to MC occurrence, and nitrogen is needed for the peptide formation of the MC structure (Graham et al., 2004; Giani et al., 2005; Wilhelm et al., 2011). The low-to-negative effect sizes of nitrogen parameters observed here may be attributed to the ability of select cyanobacterial species to use N_2-fixation (e.g., *Anabaena/Dolichospermum*, *Cylindrospermopsis/Raphidiopsis*; Chorus and Bartram, 1999). Interestingly, it was found that the correlation coefficients between total nitrogen and MC occurrence used in this study ranged from $r = 0.92$ (*study sample size* = 48), reported by Oberholster and Botha (2010), to $r = -0.69$ (*study sample size* = 12) reported by Xue et al. (2016). Comparing the cyanobacterial species composition between these studies, *M. aeruginosa*, a species incapable of N_2-fixation, dominated the system reported by Oberholster and Botha (2010), whereas the cyanobacterial bloom reported by Xue et al. (2016) shifted from *Microcystis* to two diazotrophs, *Dolichospermum* and *Planktothrix* (Pancrace et al., 2017). This is an example between two extremes, but it does reflect the importance of understanding the cyanobacterial species composition of a freshwater body in question as the nutrient requirements of species will vary over space and time.

The often weaker correlations in the reported nitrogen parameters may also be an indication that their concentrations remain at constant levels in the freshwater systems, while phosphorus concentrations vary (instances of weak total nitrogen vs. greater total phosphorus correlations observed in Islam et al., 2013; Izydorczyk et al., 2008; Rinta-Kanto et al., 2009; Yu et al., 2014; Zheng et al., 2004). Therefore, a significant correlation between MC and nitrogen would not be observed. Phosphorus often binds to sediments once entering a system (if in the presence of compounds such as iron or calcium, as well as pH and oxygen content), reducing its

bioavailability and spurring phosphorus limitation (Boyd, 2015). Nitrogen typically does not have such a mechanism (although bacterial processes do alter the structure of nitrogen, bioavailability, and concentration at times) and is frequently present at higher concentrations in freshwater systems (Guildford and Hecky, 2000; Boyd, 2015). In addition, non-linear trends between MC occurrence and nitrogen have been observed (Graham et al., 2004). Such trends are not reflected in the correlation type analyses that are routinely reported in this field. We recommend that researchers assess their data to look for non-linear trends and thresholds between environmental parameters and MC or consider making their raw data available at time of publication.

pH

Unlike temperature, little consensus exists regarding the role of pH in MC production and optimal cyanobacterial growth. For instance, laboratory studies have observed that toxic strains of *Microcystis* will have a greater cell density at both higher temperatures and pH (Watanabe and Oishi, 1985; Song et al., 1998). Yet, Cuichao et al. (2013) observed the effects of pH on the peak growth rates of *M. aeruginosa* to be more nuanced, finding that *M. aeruginosa* growth in the exponential phase benefited from a higher pH of 9.5, while a pH of 7.5 supported the greatest growth when the culture was in its stationary phase. Song et al. (1998) also found that *M. viridis* produced more MC at both a high (9.2) and neutral (7) pH, but not at moderate pH levels (7.8-8.6). The field studies assessed in this meta-analysis reveal a positive correlation between MC and pH. It is possible that higher pH conditions may allow for bicarbonate to become more bioavailable, which has shown to be favorable to phytoplankton (Boyd, 2015; Mokashi et al., 2016). Alkaline environments may also contribute to the increased occurrence of MC, as has been suggested in past laboratory studies (Watanabe and Oishi, 1985; Song et al., 1998).

Although it is generally understood, resource managers should note that cyanobacterial bloom density and the time of day during which a sample is taken will affect the reported value of pH. As phytoplankton uptake carbon dioxide during the hours and respire at night (Boyd, 2015), the presence of a substantial algal bloom will greatly influence ambient pH. The majority of studies utilized in this analysis were taken during daylight hours. In general, a further delineation of the role of pH in MC occurrence is needed, and the ability of phytoplankton to influence pH values should be reflected on by resource managers using pH in a monitoring program.

Microcystin variants used in this study

This meta-analysis took into account total MC, intracellular MC, MC-LR, intracellular MC-LR, and MC + nodularin, as these were the most reported MC variants documented in the studies that met our inclusion criterion. Extracellular MC as well as over 246 variations in the chemical structure of MC have also been documented in past studies, but are reported to a much lesser extent in the literature (Park et al., 1997; Meriluoto et al., 2017). Repeating this meta-analysis to include other MC factions or variants may alter our findings; however, the limited availability of data on these MC variants and our large sample size likely precludes significant interpretational shifts. Extracellular toxins typically occur at lower concentrations than intracellular fractions due to rapid microbial degradation, and often an excess of extracellular toxins are observed only during the decay phase of a bloom (Park et al., 1997; Zheng et al., 2004; Li et al., 2017). For instance, Park et al. (1997) monitored the intracellular and extracellular MC fractions in Lake Suwa, Japan, over four growing seasons, and observed that intracellular MC was on average ~25x higher to that of extracellular MC. Past field surveys have also documented relationships between environmental parameters and MC variants (Wu et al., 2006; Liu et al.,

2008; Li et al., 2012; Yen et al., 2012; Zhang et al., 2018). These studies have documented some variation in findings between total MC and MC variants, however, findings largely followed the same trend to that of the total MC value. Due to the robust dataset used in this meta-analysis, we expect that adding or removing MC variants or extracellular MC would not majorly affect the outcomes of this study.

Analysis boundaries and call for future analyses

The objective of this study is to provide a starting point for resource managers to determine what environmental parameters to monitor for when concerned with MC occurrence in freshwater lakes and reservoirs. We sought to distill the findings reported in a substantial number of studies in this field using meta-analysis. An attempt was made to utilize all relevant data that could be found on this topic to provide the widest picture of this issue as possible. Studies within this field incorporated a wide range of sampling and reporting methodologies, and were subsequently utilized in this analysis with limited bias. Depending on the objective of the resource manager and their specific research questions, as well as the factors affecting their system, environmental parameters useful to their sampling scheme may be different to those reported here. Further, the relationships reported here were between environmental parameters and MC value specifically and did not assess the nuisances observed in cyanobacterial bloom ecology (as mentioned throughout this study). Bloom dynamics and ecology may greatly alter what parameters, or combinations of parameters, will specifically be important to the cyanobacterial species present in a bloom. Also, the relationships drawn in this study were of areas affected by MC; findings should not be extrapolated as critical parameters to areas experiencing blooms of non-toxic phytoplankton taxa. More detailed understandings of the

drivers of blooms is critical in protecting local waterbodies and requires a concerted assessment of individual blooms.

Conclusions

Findings from this quantitative synthesis revealed environmental parameters that are significantly correlated with the hepatotoxin, MC, in freshwater lakes and reservoirs around the world. For example, eutrophic systems that are warmer, alkaline, and contain elevated concentrations of phosphorus tended to have greater MC occurrence. Such findings from a robust dataset with samples that ranged widely over both space and time reinforce the usefulness of the parameters found to be significant to be incorporated into MC monitoring programs, and managing such conditions may assist in mitigation attempts to reduce MC. Interestingly, we found most nitrogen-based parameters to be poorly or negatively correlated with MC despite this secondary metabolite containing a large proportion of nitrogen in its structure. Considering that the environmental parameters associated with significant correlations can be both directly or indirectly related to MC production (e.g., increased temperature may lead to greater toxigenic cyanobacterial biomass, pH may influence MC production or be influenced by bloom density), further laboratory and field studies that research the mechanisms that mediate interactions related to the promotion of cyanobacteria will help water resource managers better understand the conditions that contribute to the production of MC. Despite these unknowns, we contend that the parameters found significant in this analysis are the most useful to measure by resource managers when monitoring systems at risk of MC occurrence.

Acknowledgments

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Figure Legends

Figure 1.1. Locations of waterbodies from which data were obtained in this study.

Figure 1.2. Estimates of effect between microcystin occurrence and physical parameters. Estimates were converted from Fisher's z to Pearson's *r*. Studies= number of articles or datasets incorporated into the effect size estimate. I^2 = Heterogeneity (%). Error bars represent 95%

confidence intervals. * Denotes parameters with statistical significance ($p \leq 0.05$). A larger effect size box denotes a small variance within the estimate.

Figure 1.3. Estimates of effect between microcystin occurrence and biological, biochemical, or chemical parameters. Estimates were converted from Fisher's z to Pearson's *r*. Studies= number of articles or datasets incorporated into the effect size estimate. I^2 = Heterogeneity (%). Error bars represent 95% confidence intervals. * Denotes parameters with statistical significance ($p \leq 0.05$). A larger effect size box denotes a small variance within the estimate.

Figure 1.4. Estimates of effect between microcystin occurrence and nitrogen or phosphorous parameters. Estimates were converted from Fisher's z to Pearson's *r*. Studies= number of articles or datasets incorporated into the effect size estimate. I^2 = Heterogeneity (%). Error bars represent 95% confidence intervals. * Denotes parameters with statistical significance ($p \leq 0.05$). A larger effect size box denotes a small variance within the estimate. Phosphate variable includes both phosphate and orthophosphate ($p = 0.055$).

Figure 1.1

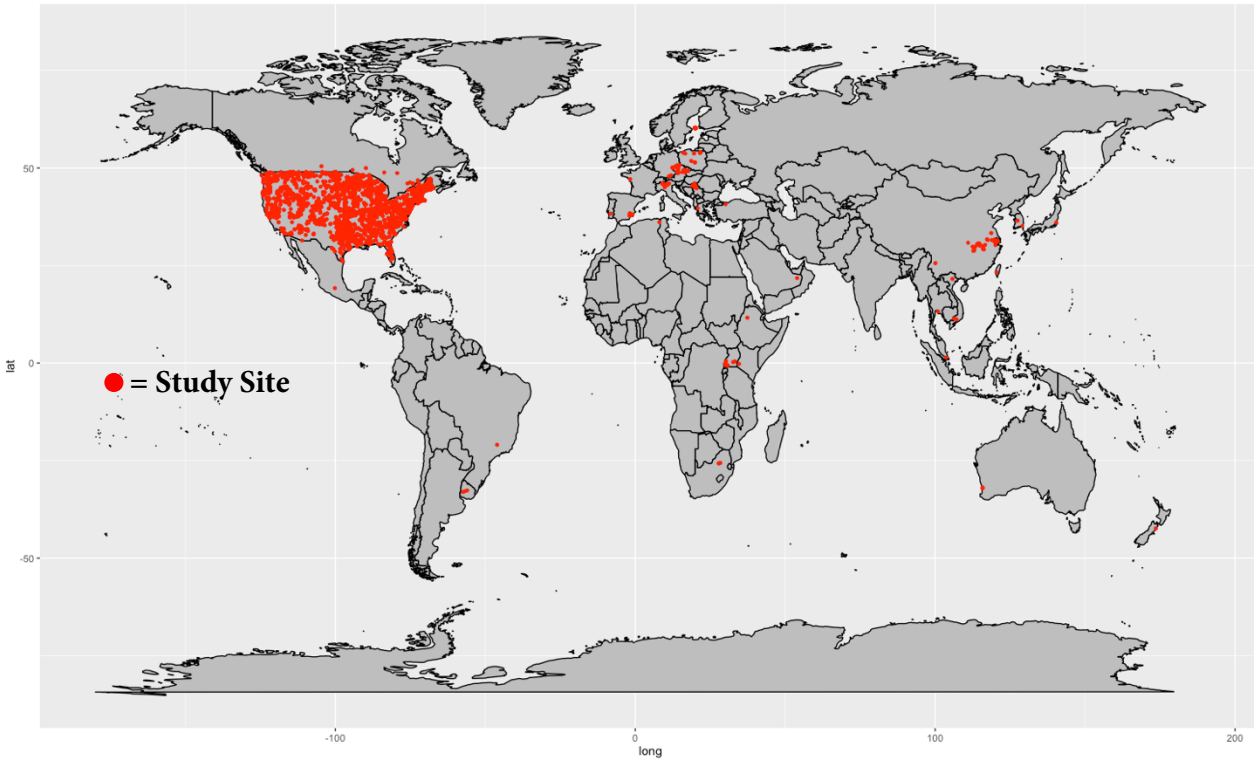


Figure 1.2

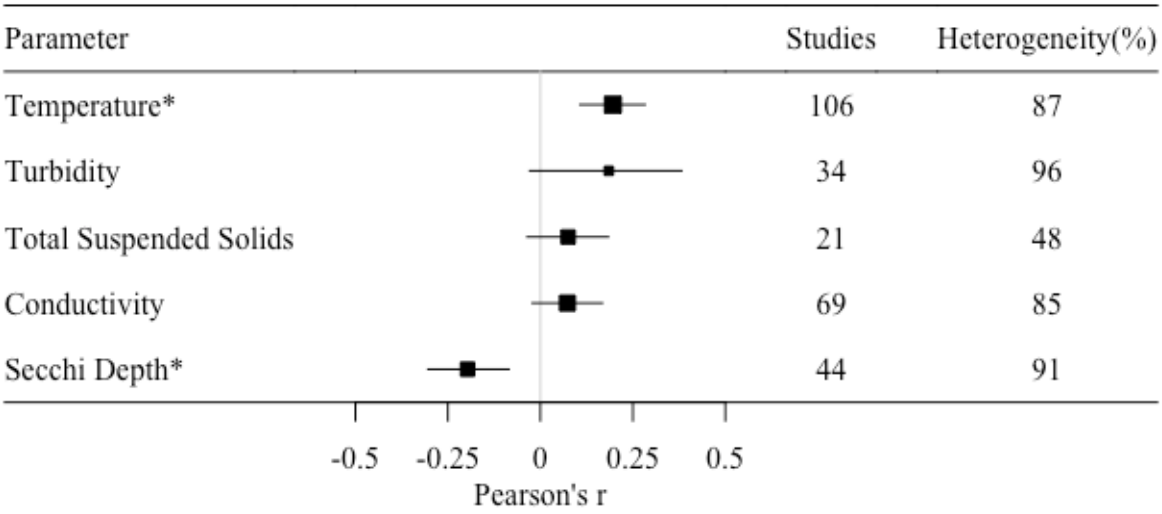

Parameter
Studies
Heterogeneity(%)
Temperature*
106
87
Turbidity
34
96
Total Suspended Solids
21
48
Conductivity
69
85
Secchi Depth*
44
91
-0.5
-0.25
0
0.25
0.5
Pearson's r

Figure 1.3

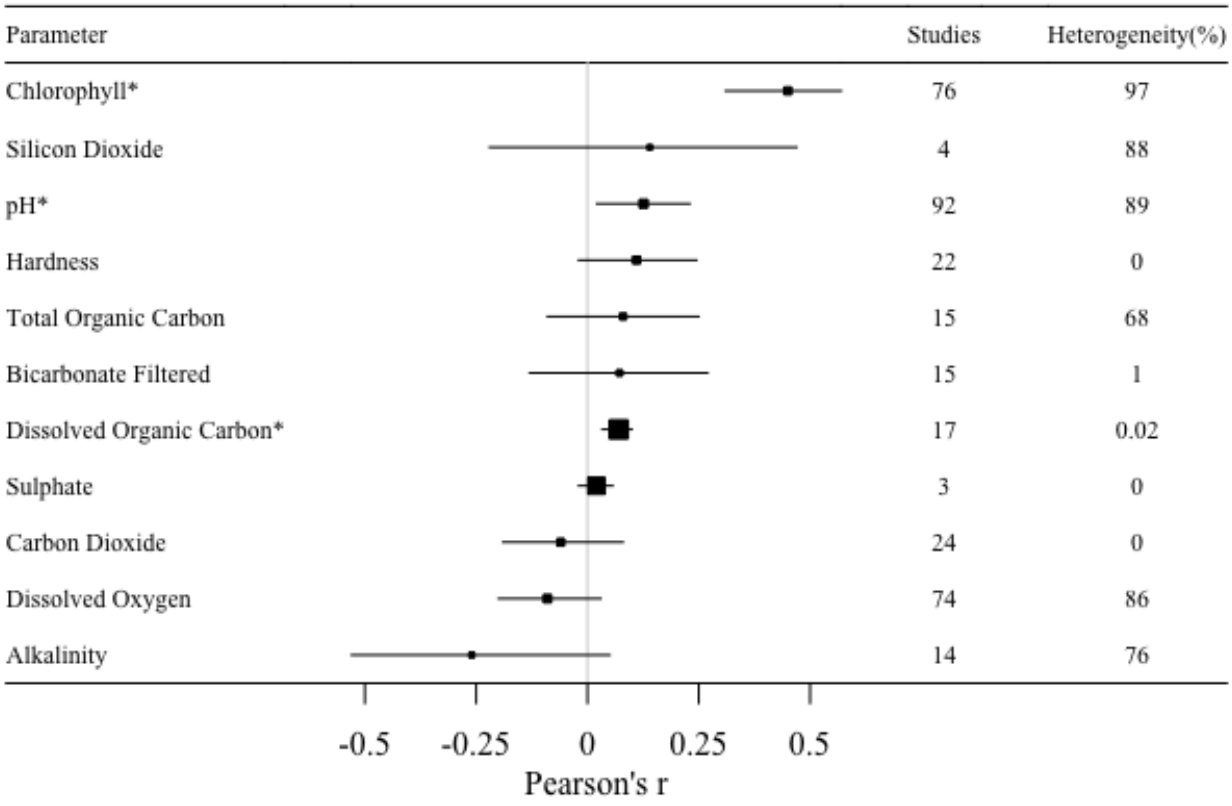
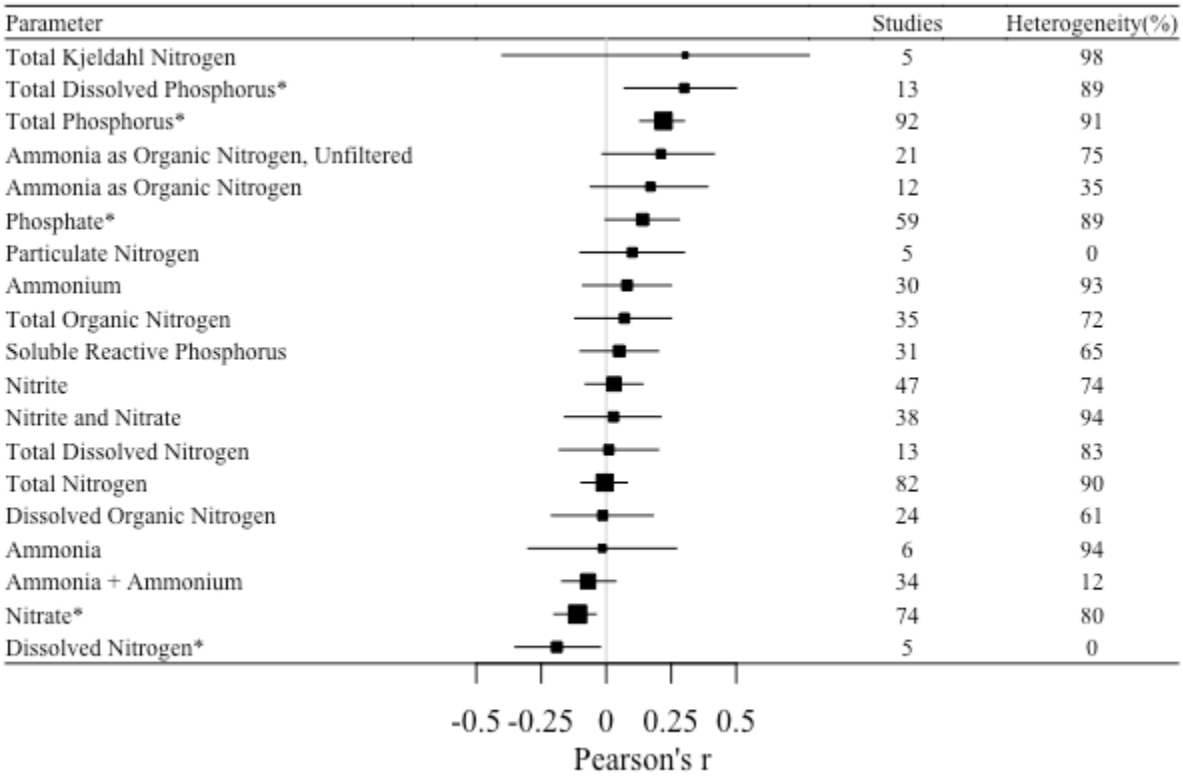

Parameter
Studies
Heterogeneity(%)
Chlorophyll*
76
97
Silicon Dioxide
4
88
pH*
92
89
Hardness
22
0
Total Organic Carbon
15
68
Bicarbonate Filtered
15
1
Dissolved Organic Carbon*
17
0.02
Sulphate
3
0
Carbon Dioxide
24
0
Dissolved Oxygen
74
86
Alkalinity
14
76
-0.5
-0.25
0
0.25
0.5
Pearson's r

Figure 1.4


Parameter
Studies
Heterogeneity(%)
Total Kjeldahl Nitrogen 5 98
Total Dissolved Phosphorus* 13 89
Total Phosphorus* 92 91
Ammonia as Organic Nitrogen, Unfiltered 21 75
Ammonia as Organic Nitrogen 12 35
Phosphate* 59 89
Particulate Nitrogen 5 0
Ammonium 30 93
Total Organic Nitrogen 35 72
Soluble Reactive Phosphorus 31 65
Nitrite 47 74
Nitrite and Nitrate 38 94
Total Dissolved Nitrogen 13 83
Total Nitrogen 82 90
Dissolved Organic Nitrogen 24 61
Ammonia 6 94
Ammonia + Ammonium 34 12
Nitrate* 74 80
Dissolved Nitrogen* 5 0
-0.5 -0.25 0 0.25 0.5
Pearson's r

Chapter 2

Generalized Additive Modeling to predict microcystin occurrence in freshwater lakes and reservoirs

Abstract

Determining the environmental conditions that influence the occurrence and concentration of the cyanobacterial toxin microcystin (MC) is a critical step for predicting cases in which the toxin will adversely affect drinking water sources, recreational waterbodies, and other freshwater ecosystems. Although widely studied, little consensus exists regarding the factors that influence MC on a global scale. The objective of this study was to identify the environmental variables most strongly associated with MC concentrations using observational data from lakes and reservoirs around the world, while also addressing the substantial proportions of missing values that such a large, aggregated dataset often involves. A total of 122 studies containing data from an estimated 2,040 lakes and reservoirs in 22 countries were used to construct a global dataset. Variables including less than 35% of non-missing observations were removed prior to analysis. Missing values for the remaining 12 predictors of MC were imputed using an iterative imputation algorithm based on a random forest approach. Variable selection was performed with generalized additive modeling on the complete case and imputed datasets. Models applied to imputed data produced lower prediction errors than those fit to the complete dataset. Variables of greatest significance to MC concentration included location (longitude-latitude pairs), total nitrogen, turbidity, and pH. Total phosphorus was not found to be a strong predictor of MC. In addition to assisting water resource managers in protecting their waterbodies against MC, the presented methodologies may provide a useful framework for future water quality modeling while accounting for varying proportions of missing data.

Introduction

Cyanobacterial blooms are a growing threat to global aquatic ecosystems due to increases in eutrophication and climate change (Paerl and Otten, 2013). Blooms can impose numerous adverse effects on these systems (e.g., hypoxia, decreased light penetration; Paerl et al., 2001; Paerl and Otten, 2013) including the production of secondary metabolites that are potentially toxic to animals and humans (i.e., also called cyanotoxins; Paerl et al., 2001; Graham et al., 2004; Malbrouck and Kestemont, 2006). Numerous cyanotoxins have been identified, including, but not limited to, dermatoxins (e.g., aplysiatoxin), neurotoxins (e.g., anatoxin, saxitoxin), and hepatotoxins (e.g., microcystin, nodularin; Sivonen, 2009). Of these cyanobacterial toxins, microcystin (MC) is routinely observed in freshwater systems experiencing cyanobacterial blooms. MC is produced through non-ribosomal peptide-polyketide synthesis in cyanobacterial strains possessing the *mcy* gene cluster (Graham et al., 2004; Hotto et al., 2008; Joung et al., 2011; Li et al., 2012) and acts as an inhibitor to type 1 or 2A protein phosphatase (Sivonen, 2009; Mankiewicz-Boczek et al., 2015). Although classified as a hepatotoxin, microcystin is known to affect the kidneys, intestines, and muscle tissues of fish and other aquatic organisms (Malbrouck and Kestemont, 2006; Martins and Vasconcelos, 2009). Over 246 variants of MC are known and toxicity between variants differs substantially (Hu et al., 2016; Li et al., 2017; Meriluoto et al., 2017). Because of its toxicity, the World Health Organization has set a recommended limit of 1 µg/L of MC in drinking water (WHO, 2003; Li et al., 2017). Although significant progress has been made in the management of MC levels in freshwater systems, MC remains a consequential topic in research, especially since the frequency and severity of toxic cyanobacterial blooms are expected to increase in the following decades (Paerl and Otten, 2013).

Field surveys relating common water quality measurements to MC occurrence have been performed during the past two decades (Kotak et al., 2000; Graham et al., 2004; Giani et al., 2005; Francy et al., 2016; U.S. Environmental Protection Agency, 2010 and 2016). Despite this, wide disparities in the environmental conditions that most influence MC production exist in the literature. For example, the documented optimal temperature range for toxigenic cyanobacteria varies widely (15-20°C, Billam et al., 2006; ~23°C, Li et al., 2017; 18-35°C, Gągała et al., 2012; and >25°C, Boutte et al., 2008). The difficulty in determining the precise relationships of environmental parameters to the production of MC stems from the complex dynamics of harmful cyanobacterial and phytoplankton blooms. For instance, multiple cyanobacterial species produce MC (*Microcystis, Anabaena/Dolichospermum, Nostoc, Planktothrix, and Nodularia*; Hotto et al., 2008), and these species have various environmental preferences and competitive traits, such as gas vesicles, nitrogen-fixation capabilities, thermal tolerances, seasonal preferences, and a wide nitrogen to phosphorus ratio tolerances, that allow for MC production in a wide range of environmental situations (Fastner et al., 2016; Paerl et al., 2001; Shan et al., 2020). The amount of MC produced per cell can also be influenced by a number of factors, including nutrient concentration (Horst et al., 2014) and temperature (Mowe et al., 2015). Such factors may influence cyanobacterial growth, but not toxin production, or vice versa. Further, a bloom dominated by a cyanobacterial species capable of producing MC will not always produce toxins, and the dominant strain must first possess the *mcy* gene to do so (Graham et al., 2004; Joung et al., 2011); a factor the can often generate water quality datasets with periods of low MC concentrations contrasted by periods of high concentrations. These numerous issues contribute to the difficulty of creating meaningful models that relate environmental factors to MC occurrence.

Although difficult, the need to identify the environmental variables most likely to influence the occurrence of MC is crucial for the management of drinking water reservoirs and other freshwater systems. Previous studies have used multi-parameter predictive and forecasting models to monitor occurrence (Giani et al., 2005; Francy et al., 2016; Otten et al., 2012; Shan et al., 2019 and 2020; Yuan and Pollard, 2017 and 2019; Francy et al. 2020). Such research often uses survey data from a single body of water or region, thereby reducing the generality of models to be used in other areas. Effective MC models have been developed utilizing both national and local waterbody datasets (Yuan and Pollard, 2019), but such research is uncommon. Contributing to the complexity of developing prediction models at larger spatial scales is securing large and spatially expansive datasets in the field of water quality, as heterogeneous studies only collect subsets of potential variables of interest and produce an aggregated dataset with varying amounts of missing values. Instances of missing data are a persistent issue observed in all fields of science, but has been readily addressed in the fields of ecology, physiology, health, and social sciences (Bennett, 2001; Wisz et al., 2008). Suggestions as to the acceptable limits of missing data within a dataset for use in modeling is a topic of debate in many fields, with the allowable limits given on a case-by-case basis (e.g., 10% allowable missingness, Bennett, 2001; 30%, Taugourdeau et al., 2014; 60%, Penone et al., 2014). Moreover, statements pointing to the importance of the heterogeneity of missing data rather than the total amount missing have been observed (Tabachnick et al., 2019). Although removing variables with large amounts of missingness (i.e., variables or datasets in which values are missing within the data matrix) may be perceived as a logical method to circumvent this issue, doing so may reduce power and introduce new bias into the developed model (Penone et al., 2014; Taugourdeau et al., 2014).

Because of these issues, limited global models using aggregated data from various publications have been employed for inference in ecology and water quality because of these issues.

Here we present novel statistical analyses to determine the water quality variables that best predict MC concentration in freshwater lakes and reservoirs globally while addressing large amounts of missing values within an aggregated dataset. We first address the issue of missing data using random forest (RF) imputation, a machine learning technique used to impute missing data without a regression model being specified (Tang and Ishwaran, 2017). RF imputation efficiently inputs unknown values within a data matrix without the use or influence of the response variable and has been used successfully in human health and biological studies (Stekhoven and Buhlmann, 2012; Penone et al., 2014). Furthermore, RF-based imputation has been shown to outperform other imputation methods for missing data (Shah et al., 2014; Kokla et al., 2019).

The effect of imputation on model fit and prediction was assessed by fitting generalized additive models (GAMs) with variable selection to the complete case and imputed datasets. GAMs account for non-normal and spatially autocorrelated data, and they accommodate nonlinear predictors and response variables by means of nonparametric smooth functions fit using regression splines (Brabec et al., 2014; Colón-González et al., 2013; Lehmann, 1998; Wood, 2017). GAMs do not have pre-defined functions to which the model has to conform, allowing for the data to determine the best-fit functions of a model (Suárez-Seoane et al., 2002). After the GAMs were fit, we then compared the predictive performance of each of the selected models on both the complete case and imputed data using 10-fold cross-validation. Lastly, the GAM with the best prediction performance, a benchmark to derive inference for management, was used to develop predictions about MC concentration from highly nonlinear data.

GAMs have been used in prior cyanobacterial studies (Carvalho et al., 2013) and other ecological studies (Lehmann, 1998; Suárez-Seoane et al., 2002), and RF techniques have been used for modeling of cyanobacterial secondary metabolites (Kehoe et al., 2015; Harris and Graham, 2017), but RF imputation has not been used in tandem with GAMs to predict MC on global scale. The use of RF imputation with a GAM for inference of nonlinear relationships may provide researchers and resource managers with meaningful insights to the production of MC in freshwater systems, even with data typically constrained by considerable missingness.

Methods

Data accumulation

Articles were retrieved in February 2018 using the Web of Science® database by combining "microcystin" with the keywords "lake", "reservoir", "environment", "parameters", "nutrients", "variables", "environmental parameters", and "environmental variables". Searches returned 3,332 articles. Studies were included in this analysis if they fit the following criteria: (1) were observational field studies (i.e., not experimental in nature), (2) from a freshwater reservoir or lake, defined as a system with little-to-no-flow (i.e., no unimpeded rivers or streams), and (3) provided numerical sampling data in figures, tables, or supplementary files. It was determined that 41 articles met these criteria (see appendix references). Data were taken directly from text or supplementary material in each article, when possible, but data were also obtained using the *metaDitigise* package in R, which allowed for the extraction of data from article figures (Pick et al., 2019). Additionally, the U.S. Geological Survey National Water Information System was used to obtain real-time data containing MC values and respective environmental parameters from 79 sites around the U.S (see appendix references). Less than (<) symbols observed throughout the USGS dataset were assigned half values. USGS dataset values that affected by

contamination (V symbol) were removed. Data were also taken from the 2007 and 2012 U.S. National Lake Assessments (NLA; U.S. Environmental Protection Agency, 2010 and 2016). Collected variables between these studies were highly similar.

In total, data from 122 studies or sites included in our analyses contained physicochemical factors (e.g., temperature; pH; Secchi disk depth, a measure of water clarity; and conductivity), nutrients (e.g., nitrogen, nitrates, nitrites, phosphorus, and phosphate), phytoplankton biomass (measured as concentration of chlorophyll (both total and -a)), and/or concentrations of MC. Many USGS sites also contained a wide array of measured variables, such as heavy metals, organic or inorganic chemicals, physicochemical measurements, and nutrients. Variables that were not also reported in the obtained published studies were largely removed, resulting in 41 predictor variables. Lastly, the reporting of MC and its variants differed between studies. The five most common MC were total MC, total MC-LR, total MC + nodularins (EPA reported data), intracellular MC, and intracellular MC-LR; these measures were kept for analysis and others were discarded. In order to maintain observation numbers and the global scale of the data, all MC data were treated as one variable. In order to maintain observation numbers and the global scale of the data, all MC concentration responses treated as one variable. Differing scales were not a concern, as MC levels from all studies were measured in µg/L. MC values larger than 500 µg/L were removed as they were considered outliers (n = 14; 0.3% of total data).

An estimated 2,040 lakes in 22 countries were represented in the global dataset (Figure 2.1). Due to the range of survey studies with differing sampling methodologies incorporated into this global dataset, varying amounts of missingness were observed within the 41 predictor variables (Table 2.1). Missingness ranged from 0% (latitude and longitude) to 99.3% (soluble reactive phosphorus). Variables not measured in at least 35% of the observations were removed

prior to statistical analysis (i.e. variables with greater than 65% of the data missing within their column), since these variables could be considered as not regularly collected in relation to MC. This left 12 remaining environmental predictor variables [Secchi disk depth, pH, ammonium, nitrite, nitrate, nitrate + nitrite, total phosphorus, organic carbon filtered, chlorophyll, total nitrogen, turbidity NTU, and specific conductivity (Table 2.2)]. Observations without both latitude and longitude coordinates were also removed, resulting in 4,316 observations from a remaining 116 studies/sites.

Statistical analysis overview

Statistical analysis of the aggregated global dataset occurred in four steps: (1) impute missing data using RF machine learning, (2) fit GAMs with variable selection on complete case and imputed data, (3) compare models fit in step 2 to both complete case and RF-imputed data using 10-fold cross-validation, and (4) fit GAM with lowest prediction errors from step 3 for inference. Each step is described in detail below. R version 4.0.2 was used to perform all statistical analyses (R Core Team, 2020).

1. Imputation of missing data using random forest machine learning

To address missingness, RF-based imputation was applied to impute missing values for the 12 predictor variables (appendix Table B1). Imputation in this study was performed by RF machine learning using the statistical package *missForest* in R, which allows for the imputation of both continuous and categorical values with limited assumptions about the data by utilizing RF machine learning (Stekhoven and Buhlmann, 2012). Briefly, the RF algorithm in *missForest* first imputes all missing values with the mean for each variable of interest in a data matrix

X = (X_1, X_2,..., X_p) with p variables, then sorts the variables X_m, m=1,..., p from least to highest proportions of missingness. Starting with the variable with the least amount of

missingness X_m, a RF is fit on the observed data values in the data matrix and is used to predict the missing values in X_m. These RF predictions are then used as the new imputed values for the missing values of variable X_m. The algorithm proceeds through the remaining predictor variables from least to highest missingness, and the algorithm repeats until a user-specified maximum number of iterations is reached or a stopping criteria is met, typically when the difference between the new imputations and previously imputed values of the data matrix X increases (Stekhoven and Buhlmann, 2012).

The response value MC was removed from the dataset before RF-based imputation to avoid biased estimates. Latitude and longitude values were also removed, since RF-based imputation methods are not suitable for imputing geolocational data. The global data matrix was then imputed, after which MC, latitude, and longitude were then returned to the dataset in their respective rows. Out-of-bag mean squared error (OOBMSE) estimates of imputation error were assessed for each of the imputed predictor variables.

2. Fitting of GAMs with variable selection on complete case and imputed data

Given that relationships between MC concentration and environmental predictors were expected to be nonlinear, GAMs of the form:

$$g\big(E(Y)\big) = \beta_0 + f_1(x_1) + f_2(x_2) + \cdots + f_{12}(x_{12}) + s(u, v)$$

were fit for the complete case data and RF-imputed data, where β_0 is the parametric intercept, $f_1, f_2, \ldots, f_{12}$ are smooth functions describing the nonlinear relationships between the 12 predictors $x_1, x_2, \ldots, x_{12}$ and the response Y, and g$(\cdot)$ represents the link function between the response and predictors (Wood, 2017). The GAMs include a spatial interaction term $s(u, v)$, where u, v are longitude-latitude pairs and $s(\cdot)$ is a two-dimensional smooth function, allowing the response to vary over space and enabling the measurement of effects of other variables to be

independent of location. Selection of terms significant to variation in MC levels was performed for each of the two GAMs using the *mgcv* package in R (Wood, 2017). A Tweedie distribution, where *mgcv* estimated the distribution parameter, and restricted maximum likelihood estimation was used for smoothing parameter estimation and variable selection. To assess fit of the models to the data, the reduced model selected for the complete case data (GAM_{CC}) and the reduced model selected using the RF-imputed data were each fit on both the complete case and RF-imputed data, and the percentage of deviance explained was recorded for each of the four fitted models. Deviance explained is approximately equivalent to unadjusted R^2 as a measure of fit for GAMs with non-Gaussian families (Wood, 2017).

3. Comparison of selected GAMs using 10-fold cross-validation

A 10-fold cross-validation of models selected for the complete case data (GAM_{CC}) and RF-imputed data (GAM_{RF}) was performed to assess the prediction accuracy of these models. Both datasets were randomly split into ten parts with nine parts used as training data and one part used as testing data. GAM_{CC} was fit on the training partitions of both the complete case and RF-imputed data, then the model fits were used for prediction on the testing portions of both datasets. The same cross-validation procedure was implemented for GAM_{RF} fit on both the complete case and imputed data. The four GAMs were compared using median absolute deviation (MAD) to assess prediction accuracy (Davydenko and Fildes, 2016).

4. Fitting of GAM with lowest prediction errors for inference

The GAM fit with lowest prediction errors determined by the cross-validation was used for inference to determine significant predictors for MC concentrations in lakes and reservoirs globally. Plots of the relationships between significant ($p < 0.05$) predictor variables determined by GAM selection and MC were generated for interpretation. Proportion of deviance explained,

a generalization of the r^2 effect size measure, was determined for each significant predictor term in the final model (Wood, 2017). The proportion of deviance explained for the variable of interest x was calculated as

$$D_x = \frac{D_F - D_R}{D_N}$$

where D_F is the explained deviance of the full GAM, D_R is the deviance of the reduced GAM with the variable of interest removed, and D_N is the deviance of the intercept-only GAM. We allowed the Tweedie distribution parameters to be estimated by *mgcv* for the full model, then used the same distribution parameters to fit the reduced and null models. Percent deviance explained ($D_x \times 100$) was reported for straight forward interpretation.

Results

Random forest imputation of the aggregated dataset exclusive of latitude, longitude, and MC concentrations generated OOBMSE for each of the 12 variables with missingness less than 65% (Table 2.2). OOBMSE varied substantially, with specific conductivity having the largest OOBMSE but also having a wide range [0 – 2,959,981.32]. However, eight out of the 12 included variables had small OOBMSE ≤ 1.46.

Variable selection on the GAM fit with the complete dataset (n = 986) removed ammonium as nitrogen and specific conductivity from the model, while ammonium as nitrogen, nitrate, and chlorophyll were dropped in the GAM using variable selection on the RF-imputed dataset (n = 4316). The complete case dataset comprised of data from the United States and Canada, while the RF-imputed dataset utilized data from all 22 countries. When broken down by latitude into temperate (30 - 60 degrees latitude north and south), sub-tropical (23 - 30 degrees north and south), and tropical (0 - 23 degrees north and south) climate regions, the amount of

data representing each region is 92.6%, 5.8%, and 1.4%, respectively. Both GAM_{CC} and GAM_{RF} had a slightly better fit (higher total deviance explained) when estimated using RF-imputed data than either of those selected models estimated using the complete case data (Table 2.3). Additionally, GAM_{CC} and GAM_{RF} fit on RF-imputed data produced lower prediction errors than those models fit on complete data, with GAM_{RF} fit on imputed data performing the best (MAD = 1.79, Figure 2.2).

The GAM_{RF} fit on the RF-imputed data, the preferred final model for inference, removed the variables ammonium, nitrate, and chlorophyll from the model ($p > 0.05$, Table 2.4). This final reduced model, given as:

$$\begin{aligned} g\big(E(MC)\big) = \; & -0.76 + f_1(Secchi) + f_2(pH) + f_3(Nitrite) + f_4(Nitrate + Nitrite) \\ & + f_5(Total\ Phosphorus) + f_6(Dissolved\ Organic\ Carbon) \\ & + f_7(Total\ Nitrogen) + f_8(Turbidity\ NTU) + f_9(Specific\ Conductivity) \\ & + s(Longitude, Latitude) \end{aligned}$$

revealed highly nonlinear relationships between the selected predictor variables and MC concentrations (Figure 2.3). Nitrite, total phosphorus, and specific conductivity had largely negative relationships with MC in the sections where the majority of their data existed (nitrite 0 - 0.1 mg/L = 99.8%; total phosphorus 0 - 2 mg/L = 99.7%; specific conductivity 0 – 5,000 mg/L = 98.7%), while the remaining variables had more nuanced relationships with MC (Figure 2.3). Total nitrogen's relationship to MC went from negative to slightly-positive from 0 - 10 mg/L, where 99.5% of the data was present. Nitrate + Nitrite had 99.8% of its values between 0 – 5 mg/L, where the relationship with MC was slightly negative. pH within the range of 6 - 10, where 99.0% of the data fell, went from a negative to positive relationship to MC with the increase as pH value increased. Dissolved organic carbon between 0 and 50 mg/L, where 99.1%

of its data were located, had a negligible relationship to MC. Turbidity measurements largely ranged from 0 - 200 NTU (99.1% of the data) and went from a negative to positive relationship to MC. Secchi disk depth had a slightly positive, but oscillating to negative, relationship to MC from 0 - 10 meters, which included 99.1% of the data. Lastly, the two-dimensional latitude-longitude smoother predicted MC to be much higher than the global average at locations within the grid of 25 - 50° longitude and 25 - 50° latitude (Figure 2.4). Location, total nitrogen, turbidity, Secchi disk depth, nitrate + nitrite, and pH had a larger association with MC than the other variables within the GAM_{RF} fit on the RF-imputed data (Table 2.5).

Discussion

Modeling of MC in freshwater systems

The development of prediction-based models to determine the occurrence of MC is an underutilized but growing practice in water resource management (Francy et al., 2016; Harris and Graham, 2017; Shan et al., 2019 and 2020; Yuan and Pollard, 2017 and 2019). Meaningful studies have been constructed assessing MC on large spatial areas and incorporating numerous waterbodies (Kotak et al., 2000; Graham et al., 2004; U.S. Environmental Protection Agency, 2016, 2010; Yuan and Pollard, 2017 and 2019; Shan et al., 2020) and have also been constructed for other cyanobacterial response variables like biomass (Carvalho et al., 2013; Vuorio et al., 2019; Shan et al., 2019 and 2020). This study is, to our knowledge, the largest determination of associative factors related to MC concentration in fresh waterbodies around the world. As such, the findings from this research may serve to support the often-anecdotal trends between MC concentration and select water quality variables and assist resource managers in determining the most relevant parameters to measure in a freshwater system experiencing MC issues.

The final model of this study utilized 12 variables that contained varying degrees of missingness up to 65%. Variables not used within our analyses merely illustrate that those variables are not commonly collected at freshwaters sites, but their exclusion in our final model is not necessarily a reflection of their lack of significance to MC production in lab or region-specific studies. For instance, temperature was reported for <35% of the data collected for our analyses, but is of noted importance to toxin-producing cyanobacteria (Billam et al., 2006; Boutte et al., 2008; Gągała et al., 2012; Li et al., 2017), as select cyanobacteria have greater growth rates at higher temperatures and prefer a more stable water column brought on by thermally stratified systems (Paerl and Huisman, 2008). This finding has been reflected in other modeling research, such as by Shan et al. (2019), who observed in a Bayesian network analysis that warmer water temperatures (≥ 24 °C) increased the probability of hazardous MC conditions (≥1.0 μg/L) occurring by 23.9%.

Limited availability is a prominent constraint in the accumulation of global data. In this work, limited relevant studies originated from the sub-tropical (30 - 23.5 ° latitude N and S) and tropical (23 - 0 ° latitude N and S) climate regions. A low number of studies in tropical regions compared to that of temperate regions is a known impediment to comprehensive inference in the field of limnology (Lewis et al., 2002; Ramírez et al., 2020). Continued assessments of water quality and MC toxicology in these areas will certainly improve our understanding of MC production in warmer climates and beyond. However, inclusion of the few currently available studies from these regions into our GAM framework, which accounts for spatial dependence, enables us to leverage information from these studies to understand average effects of environmental factors on MC concentrations across several climate regions worldwide.

Imputing missing values using random forest machine learning from original data with variables having up to 65% missingness produced models with greater predictive ability than models fit only on complete data. Random forest imputation allowed us to include a greater number of locations in the final model, resulting in a model based on data from 22 countries. Complete case data for all 41 variables was only available for two countries: the U.S. and Canada. Methods to address missing data are numerous, and limits on the total allowable amounts of missingness within each variable differs considerably even within fields, as previously described (Bennett, 2001; Penone et al., 2014; Taugourdeau et al., 2014; Tabachnick et al., 2019). Effectiveness of imputation methods vary, but select methods, including RF-based imputation deployed by the R package *missForest* used in our analysis, can impute variables with up to 60% of their original values removed without significantly altering true relationships in the data (Penone et al., 2014; Taugourdeau et al., 2014). The use of machine learning imputation, such as RF, may therefore provide a useful way to address issues of missingness in global datasets and reduce personal bias from manual removal of incomplete data.

Nonlinear relationships between MC and environmental predictors were generated using GAMs in this study. The additive structure of GAMs contributes to the interpretability of the model and makes it a preferable choice for real-world data, such as climatological and ecological research (Wisz et al., 2008; Suárez-Seoane et al., 2002; Brabec et al., 2014), and was an effective method for analyzing MC and water quality data across large spatial scales. Modest differences in the selected variables occurred in the GAMs fit on the complete case data and RF-imputed datasets. These dissimilarities are expected given the amount of missing values imputed for some variables, but overall findings regarding potential contributors to MC in freshwater lakes and reservoirs are anticipated to be conserved (Penone et al., 2014). It should be noted that the

relationships found among the studied variables and MC are independent of location, since location is included in the model. The interpretation is *ceteris paribus*, i.e., all other predictors held constant. For example, "turbidity had a nonlinear relationship to MC concentration" is true for the same lake. Variation not captured by the selected environmental variables in the final model is captured through the spatial smooth, which is a nuisance variable in our model. This is a key strength of our approach, as the effects shown in our results are average effects for any freshwater system within the spatial range of the data used in our analyses.

The concentration of data within the ranges of each of the nine environmental variables reported in Figure 2.3 should be noted (e.g., 99.5% of the total nitrogen's were from 0 - 10 mg/L). The relationship between a select parameter and MC generated outside of these ranges have much larger confidence regions due to the limited data present. Within these ranges, several trends in the data were observed and may relate to a number of different factors. Other than more obvious positive and negative relationships observed between variables and MC concentration, a flattened relationship between increasing variable values and MC concentrations may be an indication of a saturation point. For instance, Dolman et al., (2012) found that there was a saturation point between phosphorus content and cyanobacterial biovolume, while the relationship between nitrogen to cyanobacteria biovolume was not limited. Saturation of a toxic cyanobacterial bloom would eventually limit the amount of MC present within a system. Peaks in MC concentration may also reflect a limited, preferable range for toxic cyanobacteria to thrive. For instance, Graham et al., (2004) found MC concentration and cyanobacterial biomass were greatest between 1500 - 4000 µg/L. Such potential saturation values are revealed by using the GAM framework, which flexibly model these types of nonlinear relationships. Each selected variable in the final GAM and its relationship with MC will be further discussed.

Variables of significance

Nutrients: nitrogen and phosphorus

Nitrogen and phosphorus have been identified as major contributors to cyanobacterial blooms and MC occurrence (Paerl et al., 2001; Yuan and Pollard, 2017), with some models able to account for large amounts of the MC variation in US lakes using only nitrogen and phosphorus (Yuan and Pollard, 2017). In our study, total nitrogen had a largely positive relationship with MC at concentrations >1 mg/L and had the second strongest association with MC in the final GAM. Positive linear relationships between total nitrogen and MC have also been identified in past field studies (Downing et al., 2001; Pham et al., 2020) (Graham et al., 2004; data not incorporated in this study). As MC is a peptide structure, nitrogen is a key building-block in its production (Hotto et al., 2008). Nitrogen is also an integral nutrient for cyanobacterial growth and function (Hotto et al., 2008). The structure of MC comprises of 14% nitrogen, depending on variant, and conditions where the carbon to nitrogen molar ratio is below 4.3 can reduce MC production in *Microcystis aeruginosa* cultures (Wagner et al., 2019). Further, toxic *Microcystis* strains outcompete non-toxic strains in cultures containing more substantial nutrient loads (both nitrogen and phosphorus; Vézie et al., 2002). These factors contribute to nitrogen being noted as a primary correlates with MC occurrence in freshwater (Beaulieu et al., 2013; Otten et al., 2012).

Although total nitrogen had a positive relationship with MC, there were differences in the relationships with the various forms of nitrogen. Despite nitrate being non-significant in the analysis, nitrite and nitrate + nitrite displayed negative or nearly-zero associations with MC. This may suggest a nuance in best forms of nitrogen for MC production in cyanobacteria. Findings may also suggest that if toxins are at high densities, a sizable bloom would require

ample amounts of nitrogen, which could include nitrate or nitrite. Available nitrogen would be brought into the cyanobacterial cells of cyanobacteria within the bloom. If the nitrate and nitrite is within the cells, its value in the water would be low. In this way, low nitrate and nitrite values would be related to high MC.

Total phosphorus was the only phosphorus measure to be used in the final GAM and had a negative association with MC. However, 98.8% of the data was less than 1 mg/L, where the relationship between total phosphorus and MC was nearly-zero to slightly negative. This limited relationship was not expected and may be attributed to several factors. First, such a relationship may indicate that although there is evidence to suggest that phosphorus is a key nutrient to the bloom formations of phytoplankton, including cyanobacteria (Paerl and Otten, 2013; Schindler et al., 2008; Trimbee and Prepas, 1987), it is not a dominant factor in the production of MC. It has been observed in laboratory studies that MC production in *M. aeruginosa* requires a carbon to phosphorus molar ratio of less than 200, but a carbon to nitrogen molar ratio less than eight (Wagner et al., 2019). Wagner et al. (2019) suggested that phosphorus is important for cell biomass, but nitrogen has a greater importance to the production of toxins, which reflects the relatively weak association of phosphorus to MC compared to that of nitrogen observed in this analysis. Second, it could be that the sampled sites were highly eutrophic with ample amounts of phosphorus. So, a strong relationship between phosphorus and MC would not be observed. The average phosphorus content of the sampled lakes before the imputation of the dataset was 119.79 ± 309.08 µg/L, which equates to conditions of possible hypereutrophy based on the Carlson Trophic State Index (Carlson and Simpson, 1996). Such eutrophic, phosphorus-rich conditions have been shown to have other nutrients, such as nitrogen, be the dominant contributors to cyanobacterial bloom formation and/or the production of MC (Scott et al., 2019).

The reduction of both nitrogen and phosphorus will likely be needed to reduce cyanobacterial blooms and subsequent MC toxins. This has been put forward in prior modeling studies such as Shan et al. (2020), whose Bayesian analysis identified that reducing MC risks in three lakes in China could be achieved by setting total phosphorus and nitrogen thresholds of 0.5 mg/L and 1.8 mg/L, respectively. Such findings solidify the growing call for dual nitrogen and phosphorus reductions in systems plagued by cyanobacterial blooms (Paerl and Otten, 2013). Further the need for a better understanding of nutrient speciation, timing, and magnitude into a freshwater system and how this affects cyanobacterial and algal blooms is vital (Gilbert, 2017).

pH

The majority of pH values used in this study were between 6 - 10, where the relationship of pH to MC turned from negative to positive with increased pH value. The increase in MC production at more alkaline pH concentrations has been documented (Song et al., 1998), and MC-producing species have been shown to outcompete other phytoplankton in prior laboratory studies (Yang et al., 2017). Because of the ease of measuring pH with handheld meters, it is a useful measurement to track cyanobacterial bloom formation and possibly MC occurrence. However, it should be noted that bloom densities and sampling time may affect the value of pH within a system. Typically, pH values will be higher in the daylight hours as primary producers photosynthesize and lower at night when respiration occurs. Variations in pH values are also affected by the alkalinity (i.e., buffering capacity) in a waterbody (Boyd et al., 2016). If using pH as a measurement for potential MC occurrence, we recommend measurements be taking at a consistent time between sampling events, and managers should also be tracking phytoplankton bloom density and factures such as alkalinity.

Specific Conductivity

Specific conductivity had a slightly negative relationship with MC from 0 - 5,000 µS/cm, where 98.7% of the data fell. Laboratory studies have indicated that both the growth of cyanobacteria and microcystin concentration decreases with increased salinity (Georges des Aulnois et al., 2019). Moreover, the MC-producing species, *Microcystis*, has been found to have a salinity tolerance of up to 2 Practical Salinity Units (PSU), while other species can have salinity limits of 35 PSU or greater (Paerl et al., 2001). Specific conductivity measures a range of salts and inorganics capable of holding an electrical current, however, such compounds may hold differing importance to MC production. For instance, Cerasino and Salmaso (2012) showed positive correlation to microcystin (Spearman correlation= 0.50) in Italian sub-alpine lakes, while Aboal and Puig (2005) showed a negative correlation (Pearson correlation = -0.31) in the reservoirs of the Segura river basin of SE Spain. Because of the variation in past findings and the wide interpretabilities of specific conductivity itself, further research is needed to understand the specific relationships that conductive compounds have with MC occurrences, and specific conductivity's usefulness in monitoring MC should be assessed on a case-by-case basis.

Dissolved organic carbon

Dissolved organic carbon had an overall nearly-zero to negative relationship with MC from 0-100 mg/L, where most of the data were present. This relationship may be indicative of conditions not favorable for cyanobacterial growth or MC production, although a beneficial relationship between cyanobacterial growth and dissolved organic matter has been observed previously under laboratory conditions (Paerl et al., 2001; Zhao et al., 2019). This is due to the ability of cyanobacterial cells to directly uptake this carbon source for growth or to be used by bacterial communities supporting the cyanobacterial blooms (Paerl et al., 2001; Znachor and Nedoma, 2010). Such carbon and organic matter can be from both allochthonous and

autochthonous sources (Wilkinson et al., 2013), although a deletion between such carbon sources was not made in this study. More dissolved organic matter is typically present in a system as cyanobacterial blooms decay and cells lyse (Tessarolli et al., 2017). The negative relationship observed between dissolved organic carbon and MC in this study may be indicative of the break-down of a bloom and therefore a reduction in the production of MC. Yet, this relationships was slight and could suggest blooms that were stable, with both active and declining bloom components co-occurring.

Transparency: turbidity and Secchi disk depth

Turbidity had a nonlinear relationship to MC, with a positive relationship occurring around ~50 - 200 NTU. The relationship between MC concentration and turbidity has been observed in past lake surveys. Kotak et al. (2000) suggested that MC was produced at low light intensities, thereby making turbid systems favorable. Increased MC production at low light intensities has been reported in laboratory culture studies using *Microcystis* (Wiedner et al., 2003), but toxic strains of *Microcystis* do not produce more MC at low- versus high-light conditions. However, toxic strains will dominate non-toxic strains under both light conditions (LeBlanc Renaud et al., 2011). *Microcystis* colonies are equipped with gas vesicles, allowing them to regulate buoyancy (Paerl and Otten, 2013). This physiological characteristic may allow them to form blooms on the surface, giving them preferential access to sunlight over other phytoplankton species and non-toxic strains of cyanobacteria by circumventing the low-light conditions of highly turbid areas.

Secchi disk depth, a measure of water transparency, had an oscillating relationship with MC. Approximately 99.2% of the Secchi depth measurements in the data were less than 10 m, in which a peak of positive relationship was observed from ~2-4 m before returning to a negative or

nearly-zero relationship on either side of this range. In general, MC was unrelated to Secchi disk depth as its value increased. This was expected, as greater Secchi depths can be associated with oligotrophic conditions containing low phytoplankton densities (Carlson and Simpson, 1996). Secchi disk depth is a useful tool to track the progression and density of the bloom in some situations (Joung et al., 2011). However, measurements are not always proportional to cyanobacterial or phytoplankton abundances in a waterbody as inorganic turbidity (e.g., sediment) or other pollution factors may affect Secchi depth values (Swift et al., 2006). Such disruptive factors may have contributed to the minimal relationship between Secchi depth and MC, and may be the reason for the negative relationship between MC and Secchi depths < 2m.

Location: latitude and longitude

Location was the most important predictor of MC. In a study of 200 Midwestern U.S. lakes and reservoirs, particulate MC was significantly correlated to an increase in latitude, which was attributed to correlated changes in nutritional, physical, and chemical parameters (Graham et al., 2004). An assessment of microcystin concentration over the contiguous U.S. by grouping land patterns into nine ecoregions also found substantial differences in the microcystin concentrations among regions (Beaver et al., 2014). In this study, MC was predicted to be highest at around 25 - 50° longitude and 25 - 50° latitude. This corresponded to some of the highest MC concentrations documented in this study found in eastern Europe, including Serbia (Simeunovic et al., 2010; Taugourdeau et al., 2014) and Poland (Mankiewicz-Boczek et al., 2006). As data collected for this study largely originated in North America, additional MC data collection in Asia, Europe, and Africa may better help determine global factors contributing to MC occurrence and accumulation.

Conclusion

The results of multiple statistical methods incorporated into this study revealed various environmental variables significantly related to the concentration of MC globally and included a technique to impute missing data within an aggregated water quality dataset. To our knowledge, the aggregated dataset is the largest global accumulation of MC data. Not only did the models fit on imputed data produce more accurate predictions, but the imputed data also provided more spatial coverage for the model. Using datasets with low overall missingness is recommended, but some missingness may be resolved using imputation and will likely need to be assessed on a case-by-case basis by using metrics like parameter significance and MAD value to determine if findings are accurate.

The final GAM indicated that environmental variables, such as location, total nitrogen, turbidity, and pH, are associated with MC concentrations in fresh waterbodies. Numerous nonlinear relationships were observed between the selected predictors and MC concentration. The significance observed in our results reflect the usefulness of GAMs to assess nonlinear and non-normal data. These findings may serve as both reference and validation to often varying summaries of the trends in MC concentration worldwide. Moreover, the combination of machine learning methods for imputation and nonparametric modeling used in this analysis may serve as an effective procedure for using global datasets containing large amounts of missingness, which is often the case when using data from many origins and sampling methodologies.

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Tables and Figures Legends

Table 2.1. Ratio of missingness (% missingness) in the variables collected for this analysis.

Table 2.2. Out of box mean squared error (OOBMSE) for random forest imputation for 12 predictor variables.

Table 2.3. Total deviance explained (%) for variable selection on GAMs when estimated using complete and RF-imputed datasets. GAM_{CC} is the selected model from completed case data; GAM_{RF} is the selected model from RF-imputed data.

Table 2.4. Summary of final GAM fit on RF-imputed data (n = 4316). Deviance explained = 62.5%.

Table 2.5. Percent deviance explained ($D_x \times 100$) for each of the selected predictor variables in the final GAM fit on RF-imputed data.

Figure 2.1. Locations of waterbodies from which data were used in this study.

Figure 2.2. Median absolute deviation (MAD) from 10-fold validation of reduced models selected by generalized additive models (GAMs) fit on complete data and RF-imputed data. GAM_{CC} is the selected model from complete case data; GAM_{RF} is the selected model from RF-imputed data.

Figure 2.3. Centered smooths of variables selected by the final GAM fit on RF-imputed data. Black hash marks represent the presence of data for the x-axis variable.

Figure 2.4. Centered MC concentrations estimated by the final GAM fit on RF-imputed data using two-dimensional smoothing over longitude-latitude coordinates. Black points represent true locations from data.

Table 2.1

Variable	Ratio of missingness
Soluble Reactive Phosphorus	99.3
Phosphate/Orthophosphate as Phosphorus	99.1
Dissolved Inorganic Nitrogen	99.1
Particulate Nitrogen	98.7
Chlorophyll Corrected	97.9
Carbonate	97.6
Alkalinity	97
Bicarbonate	96.9
Phosphate/Orthophosphate Unfiltered	96.8
Ammonium as Organic Nitrogen	95.6
Dissolved Solid	95.6
Turbidity NTRU	95.4
Total Dissolved Nitrogen	94.5
Hardness	93.7
Carbon Dioxide	92.9
Turbidity FNU	92
Suspended Sediments	91.7
Organic Nitrogen Filtered	91
Total Suspended Solids	90.8
Total Dissolved Phosphorus	90.5
Ammonium as Organic Nitrogen, Unfiltered	88.5
Ammonia and Ammonium	85.6
Total Organic Nitrogen	84.9
Phosphate/Orthophosphate	83.5
Dissolved Oxygen	75
Temperature	68.4
Organic Carbon Unfiltered	65.5
Nitrite	58.7
Turbidity NTU	42.1
pH	41.5
Organic Carbon Filtered	36.2
Ammonium	36.2
Secchi Disk Depth	29.9
Nitrate and Nitrite	24.5
Nitrate	23.5
Chlorophyll	22.6
Specific Conductivity	19
Total Nitrogen	18.6
Total Phosphorus	18.5
All microcystin	0
Latitude	0
Longitude	0

Table 2.2

Variable	OOBMSE
Secchi Disk Depth	1.31
pH	0.22
Ammonium	0.01
Nitrite	0.00
Nitrate	0.70
Nitrate and Nitrite	0.72
Total Phosphorus	0.07
Organic Carbon Filtered	142.48
Fluorometric Chlorophyll	1897.74
Total Nitrogen	1.46
Turbidity NTU	686.39
Specific Conductivity	2,959,981.32

Table 2.3

		Data	
		Complete case	RF-imputed
Model	GAM_{CC}	60.7	62.5
	GAM_{RF}	58.2	62.3

Table 2.4

Parametric	Estimate	Standard error	t-value	p-value
(Intercept)	-0.76	0.02	-33.97	< 0.0001
Approximate significance of smooth terms				
	edf	Ref. df	F	p-value
Longitude and Latitude	27.23	29	61.52	< 0.0001
Secchi	8.22	9	11.51	< 0.0001
pH	5.07	9	16.07	< 0.0001
Ammonium	0.00	9	0.00	0.563
Nitrite	4.84	9	2.59	< 0.0001
Nitrate	0.42	9	0.07	0.196
Nitrate + Nitrite	5.59	9	10.95	< 0.0001
Total Phosphorus	6.80	9	5.10	< 0.0001
Dissolved Organic Carbon	7.17	9	5.66	< 0.0001
Fluorometric Chlorophyll	1.02	9	0.22	0.125
Total Nitrogen	6.22	9	34.69	< 0.0001
Turbidity NTU	8.43	9	22.51	< 0.0001
Specific Conductivity	2.16	9	1.78	< 0.0001

Table 2.5

Variable	Deviance explained (%)
Latitude and Longitude	9.01
Secchi	0.71
pH	1.13
Nitrite	0.10
Nitrate + Nitrite	0.08
Total Phosphorus	0.14
Dissolved Organic Carbon	0.31
Total Nitrogen	1.80
Turbidity (NTU)	1.15
Specific Conductivity	0.00

Figure 2.1

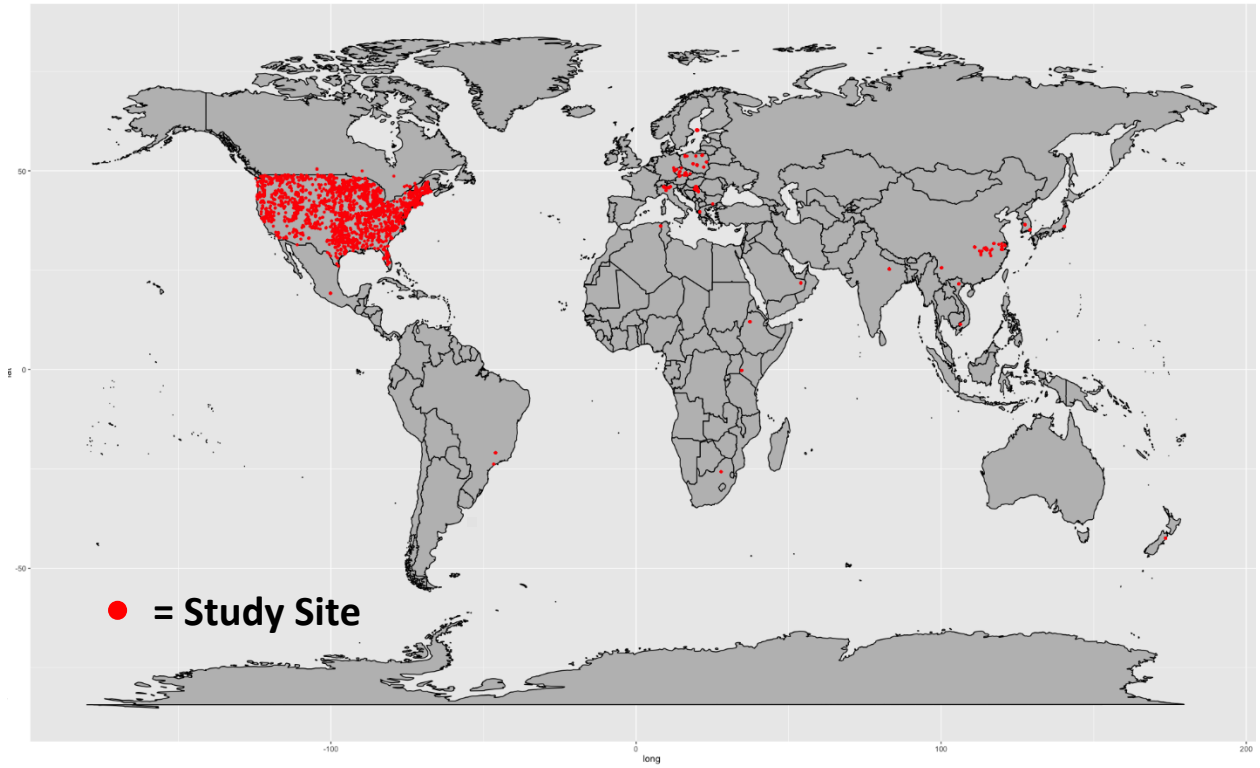


Figure 2.2

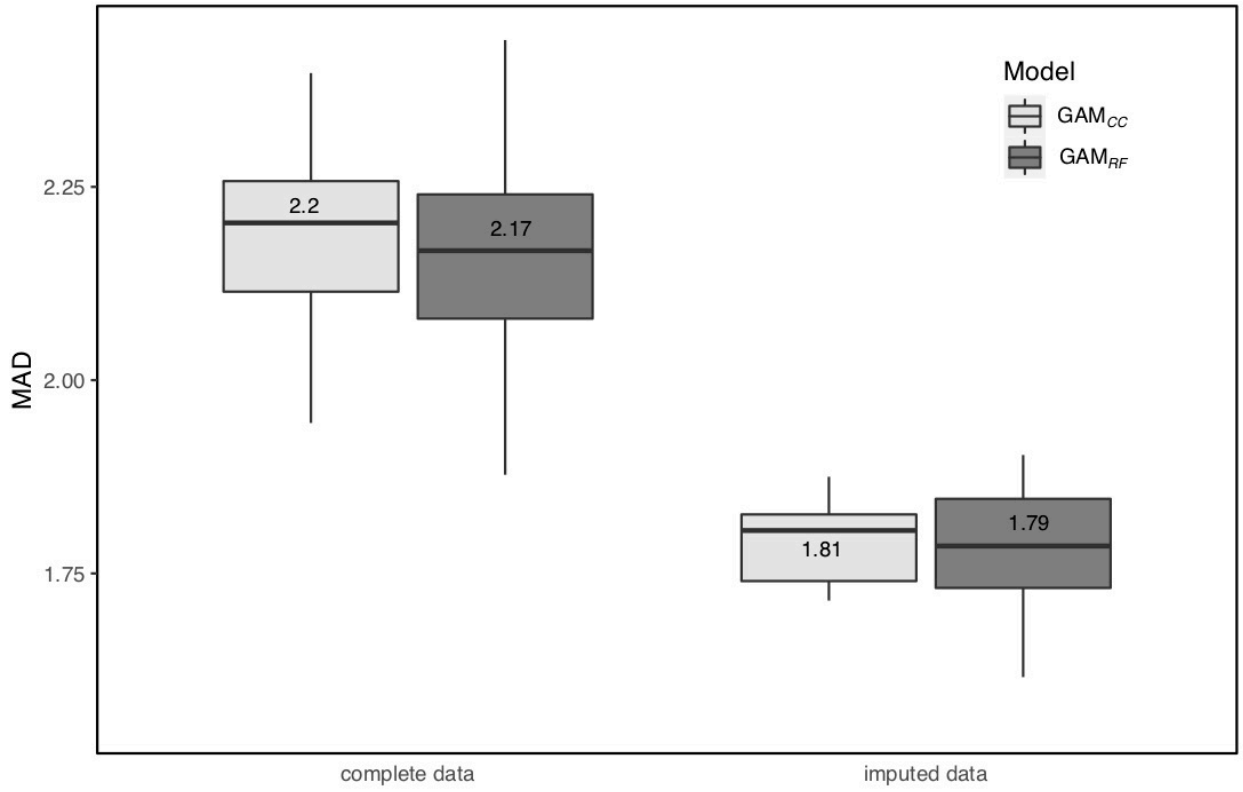
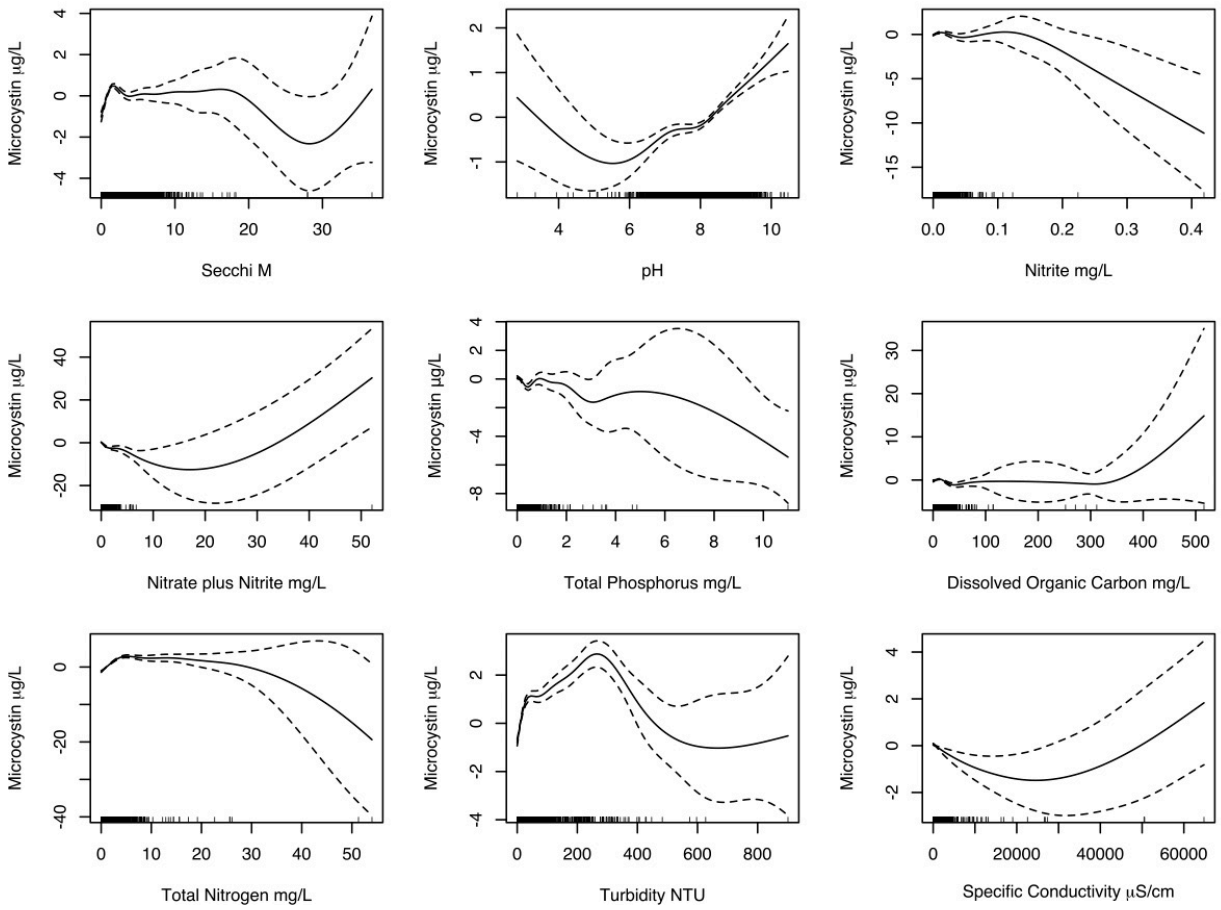
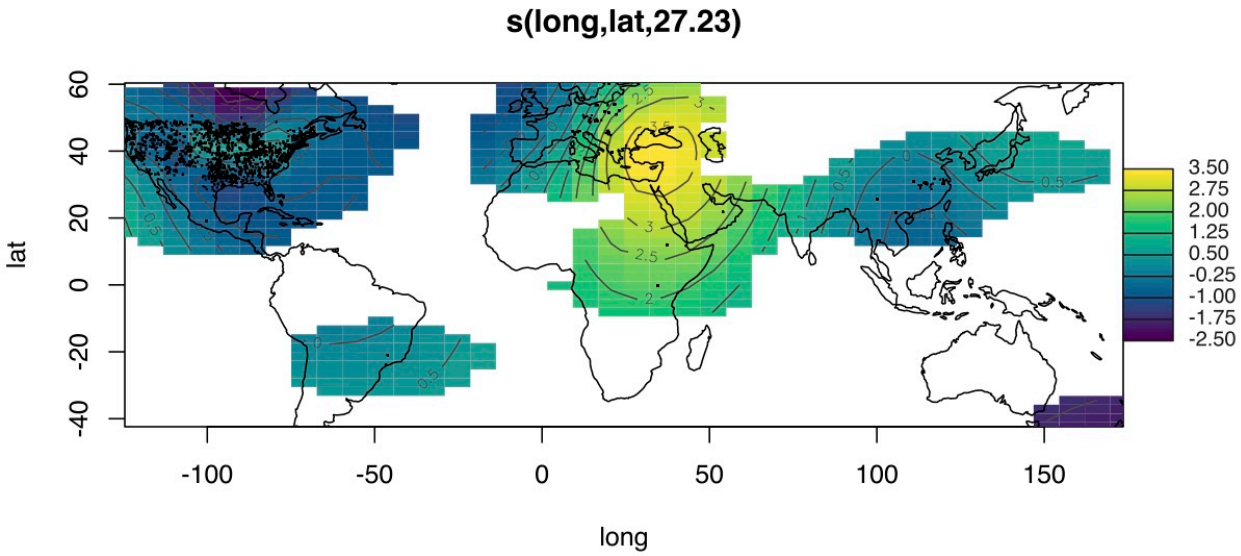

Model
GAM$_{CC}$
GAM$_{RF}$
MAD
2.25
2.00
1.75
2.2
2.17
1.81
1.79
complete data
imputed data

Figure 2.3


Microcystin µg/L
Secchi M
pH
Nitrite mg/L
Nitrate plus Nitrite mg/L
Total Phosphorus mg/L
Dissolved Organic Carbon mg/L
Total Nitrogen mg/L
Turbidity NTU
Specific Conductivity µS/cm

.

Figure 2.4


s(long,lat,27.23)
lat
60
40
20
0
-20
-40
-100
-50
0
50
100
150
long
3.50
2.75
2.00
1.25
0.50
-0.25
-1.00
-1.75
-2.50

Chapter 3

Field evaluation of seven algaecides to control cyanobacterial blooms in aquaculture

Abstract

Harmful algal blooms negatively affect water quality in hypereutrophic systems such as those common in aquaculture. However, few algaecides are approved for use in food-fish aquaculture. This study assessed the effectiveness of seven products, including hydrogen peroxide (as a concentrated liquid or in granular form [PAK-27]), peracetic acid (as VigorOx SP-15 and Peraclean), copper (as copper sulfate in unchelated [powder] or chelated [Captain] forms), and a clay-based product (as Phoslock) on phytoplankton (including cyanobacteria) and zooplankton biomass. Specifically, we sought to identify treatments that could effectively reduce cyanobacterial density. Each products was tested in a 14-day laboratory and 35-day field experiment to assess their short- and long-term performance. Although some products (i.e., copper-based products and liquid hydrogen peroxide) quickly reduced phytoplankton density, effects were short-lived given that chlorophyll concentrations returned to starting concentrations within 21 days. In contrast, all but one product (i.e., concentrated liquid hydrogen peroxide) maintained low phycocyanin concentrations for 35 days after initial treatment. Zooplankton biomass demonstrated large, negative effects for most algaecides; however, zooplankton returned to pretreatment densities for most treatments except copper-based algaecides. In general, copper-based products remain the most efficient and economical choice to reduce total phytoplankton biomass in aquaculture systems. However, peracetic acid-based products effectively and quickly reduced cyanobacteria while having marginal effects on beneficial algae and zooplankton. Such algaecides could be effective alternatives to copper-based products depending on the outcomes sought by aquaculture farmers.

Introduction

Harmful algal blooms negatively affect water quality in freshwater, estuarine, and marine systems around the world (Chislock et al., 2013a and b). Such events are more common, extreme, and persistent in nutrient-rich systems like those found in aquaculture (Schrader et al., 2018; Tucker et al., 2020). Algal blooms often create anoxic or hypoxic conditions under periods of low light or as cells decay associated with microbial degradation. In intensive aquaculture systems, daily pond aeration is often required to maintain safe dissolved oxygen concentrations, which increases production costs. Secondary metabolites of toxigenic phytoplankton, such as microcystin which is a class of hepatotoxins produced by some genera of cyanobacteria (blue-green algae), may affect the liver, spleen, and kidneys of fish facilitating sub-chronic issues (e.g., reduced growth and feeding, deformities, increased cortisol levels; Malbrouck and Kestemont, 2006), or, in extreme situations, induce acute die-offs (Zimba et al., 2000). Moreover, some cyanobacterial genera can produce off-flavor compounds (e.g., 2-methylisoborneol (MIB), geosmin), which are non-harmful (Dionigi et al., 1993) but generate unwanted taint in fish fillets. This issue costs the U.S. catfish aquaculture industry an estimated $23 million annually due to lower market prices, prolonged holding times (for purging off flavor), and extended feeding (Hanson, 2003).

To combat issues generated by cyanobacterial blooms, aquaculture relies primarily on the use of chemical controls due to their effectiveness in rapidly reducing phytoplankton biomass (Bosma and Verdegem, 2011; Schrader et al., 2005; Viriyatum and Boyd, 2016). Past research has shown that a number of algaecide types can control nuisance algal blooms in nutrient rich environments similar to that of farm-pond aquaculture (Sinha et al., 2018; Schrader et al., 2005; Barrington et al., 2013; Bishop and Richardson, 2018). For example,

copper sulfate ($CuSO_4$) can reduce excessive algal growth in ponds with moderate risk to the farmed fish when used appropriately, such as testing ambient alkalinity prior to treatment (Viriyatum and Boyd, 2016). Despite this, there is concern that chemicals, such as heavy metals like copper, may persist in the environment for extended durations, could have negative effects on non-target organisms, and may require repeated applications to prevent bloom resurgences, thus increasing water quality management costs and toxicity risks (Viriyatum and Boyd, 2016). Only two algaecides are approved for algal bloom control in aquaculture (i.e., $CuSO_4*H_2O$ and Diuron [phenylurea-based herbicide-turned-algaecide product; to be used specifically for the control of cyanobacteria that produce MIB; EPA 2003]). Such a limited variety of chemical controls is possibly due to the requirements needed to receive the U.S. Environmental Protect Agency's (EPA) approval under the Federal Insecticide Fungicide and Rodenticide Act (FIFRA; Laughinghouse et al., 2020). Further, $CuSO_4$ is a cost-effective, low volume method to reduce cyanobacteria, and issues of toxicity can be mitigated by utilizing repeated doses (Tucker et al., 2005).

Despite the limited number of approved algaecides, recent research has identified numerous chemicals that can effectively reduce cyanobacterial biomass, including chelated copper (Bishop et al., 2017), granular (sodium carbonate peroxyhydrate) and liquid hydrogen peroxide (Sinha et al., 2018; Yang et al., 2018), and peracetic acid (Enviro Tech, 2003). Clay compounds have also been identified as means to bind to cyanobacteria for removal (Lu et al., 2017) and/or by binding to phosphorus to reduce nutrient availability to blooms (Bishop and Richardson, 2018). Despite a large amount of literature on the subject, research on the effectiveness of a specific algaecide is often context-specific considering each study is conducted under disparate conditions with varying cyanobacterial genera dominating the

system, thus leading to a dissonance in findings between studies. Such variation in results is more pronounced when experiments compare results across algaecides (Sinha et al., 2018) or attempt to extend results from the lab to the field (Yang et al., 2018). For example, Yang et al. (2018) observed that hydrogen peroxide (H_2O_2) in liquid form under uniform laboratory conditions was effective at eliminating *Dolichospermum* (formerly known as *Anabaena*), *Cylindrospermopsis*, and *Planktothrix*, but was less effective at reducing *Microcystis*. Furthermore, the prolonged effectiveness of a treatment is questionable as many are assessed for short durations (<7 days; Barrington et al., 2013; Greenfield et al., 2014). Such differences in experimental design between published studies may lead to varying outcomes and subsequent inaccurate perceptions of the effectiveness of a product to reduce nuisance cyanobacterial blooms.

In general, few studies have tested multiple algaecides in a single study under uniform conditions (refer to Sinha et al., 2018). The purpose of this study was to compare the effectiveness of $CuSO_4$, as it is the only fully EPA approved algaecide for use in food-fish aquaculture to six other algaecides to control blooms of phytoplankton, specifically cyanobacteria, in the field. This study assessed the effectiveness of seven algaecides including, $CuSO_4$, Captain® (chelated copper), PAK-27® (sodium carbonate peroxyhydrate, H_2O_2-based), liquid H_2O_2, VigorOx SP-15® (peracetic acid), Peraclean® (peracetic acid), and Phoslock® (modified clay for phosphorus binding, not an algaecide as the others, but hereby referred to as an ‘algaecide’ or ‘product’ to maintain uniformity; Appendix Table C1). The products were initially tested across a broad range of concentrations in a 14-day laboratory-based microcosm experiment to identify target concentrations for each algaecide in a

subsequent 35-day field mesocosm experiment where effects on phytoplankton and zooplankton biomass were assessed.

Methods

Laboratory experiment

Shoreline pond water samples were collected using buckets from three active catfish aquaculture ponds experiencing cyanobacterial blooms (dominated by *Microcystis*) on the E.W. Shell Fisheries Center of Auburn University, AL during May 2019. The pond water was combined in equal parts, returned to the lab, filtered through a 500 µm mesh to remove large debris, and placed into an acid-washed bucket. To supplement phytoplankton densities, BG-11 media (Rippka et al. 1979) was stirred into to the combined pond water such that media comprised 10% of the total volume. The final phytoplankton biovolume of the mixture comprised of cyanobacteria (91.5%), green algae (7.5%) and diatoms (1%). Of the cyanobacteria, *Microcystis* (79.1%), *Raphidiopsis* (11.3%), and *Oscillatoria* (5.9%) were the most dominant. The mixture was then distributed to 87, 500 mL glass jars each, to a volume of 435 ml. Jars were capped and mixed before collecting A/E filtered samples for a measurement of two algal pigments, chlorophyll (measure of total phytoplankton abundance) and phycocyanin content (measure of cyanobacterial abundance), that were measured using fluorometry (Turner Designs Trilogy®). Chlorophyll was determined by extracting filters in 90% ethanol for 24 hours at 4 ºC (20 mL pond water; Sartory and Grobbelaar, 1984). Phycocyanin was measured by extracting filters in a 50 mM phosphate buffer (Ricca Chemical ®) for four hours in the dark (20 mL pond water; Kasinak et al., 2014). After collecting initial algal pigment samples, 395 mL of pond water remained in each jar.

Jars were then dosed with one of seven products (Appendix C; Table C1). Each product was tested at four different treatment concentrations with three replicates for each concentration. Secondary stocks of each chemical were made with DI water at a concentration such that each jar received a 5 mL addition of the secondary stock to achieve the required chemical dosage (total jar volume now 400 mL). Control jars received 5 mL of DI water containing no chemicals. Phoslock treatments were based on the amount of total phosphorus present within a water body. As such, total phosphorus was measured for the pond water and BG-11 mixture before the treatment using a colorimetric assay spectrophotometry (Gross and Boyd, 1998) and found to be 2.2 mg/L. Control jars that received no chemical additions were also included, and received 5 mL of DI water to maintain uniformity between jars. After the 5 mL of the secondary stocks were added, jars were then inverted 3 times, their caps loosened, and incubated at 30 °C on an 8 hr light: 16 hr dark schedule (florescent lighting; intensity = 80 µmol/m^2/s).

The laboratory experiment lasted for 14 days. Jars were mixed by inverting 3 times and rotated within the incubator (Percival® model I-36VL) daily to minimize light variation across jars. Algal pigment measurements were collected via pipette on days 0, 1, 3, 5, and 7 (20 mL for both chlorophyll and phycocyanin). A repeated-measures analysis of variance (RM-ANOVA) using a restricted maximum log-likelihood (REML) method was used to assess differences in total phytoplankton (chlorophyll) and cyanobacterial (phycocyanin) densities over time. Tukey's multiple comparison tests were used to compare mean effects among treatments. The analysis was performed using the *nlme* package in R (Pinheiro et al., 2020). The lowest concentration of each product that clearly and effectively reduced total phytoplankton (using chlorophyll values) and specifically cyanobacterial biomass (using phycocyanin values) to that of the control was selected for use in the field experiment.

Field experiment

The field experiment was conducted during June 2019 in a 22-acre earthen aquaculture pond containing hybrid catfish (blue x channel catfish; *Ictalurus punctatus* x *I. furcatus*) housed within an in-pond raceway system at the E.W. Shell Fisheries Center of Auburn University, AL (S1; Boyd and Shelton, 1984). Each product was tested in three, randomized replicate mesocosms, and the control had four replicates (25 mesocosms in total). Mesocosms were cylinder-shaped and made of greenhouse plastic (1310 L volume) that were sealed at the bottom and open at the top and suspended to a floating dock positioned in the center of the pond (Figure 3.1). Mesocosms were filled using a pump (~300L/min) with surrounding pond water after being sieved through 200 µm mesh to exclude large debris but to include ambient zooplankton and phytoplankton. Prior to filling, the pond was sampled for total nitrogen and phosphorus [both measured using persulfate digestion and spectroscopy (Gross and Boyd, 1998)]. Based on these values, potassium phosphate (K_2HPO_4) and potassium nitrate (KNO_3) were added to each mesocosm to reach concentrations of 2.6 mg/L total nitrogen and 0.22 mg/L total phosphorus, as a means to facilitate phytoplankton growth. Mesocosms were then left for 11 days to allow phytoplankton abundance to increase and stabilize.

On day 0 (11 days after filling and fertilizing), two integrated vertical water samples were obtained using a rigid tube sampler (inside diameter = 51 mm) to a depth of 1 meter (4 L of sample collected total). Samples were combined in a bucket and placed into a plastic cube container. Water samples were returned to the lab to be processed for chlorophyll and phycocyanin pigments, as well as for phytoplankton and zooplankton diversity and abundance. Phytoplankton samples were preserved using 1% Lugol's iodine solution. The preserved samples were then settled in a Hydrobios settling chamber and enumerated on an inverted microscope by

counting cells observed in 25 fields from 100x-400x (Yang et al., 2018). Zooplankton from 2 L samples were collected on a 100 µm filter and preserved in 95% ethanol before enumeration in a Sedgewick-Rafter chamber on a compound microscope by counting all zooplankton observed at 100x (Yang et al., 2018). Phytoplankton and zooplankton were identified using Edmondson (1959). Phytoplankton were identified to the genus level. Zooplankton were identified to the sub-order or genus. Dominant phytoplankton included those in the grouping chlorophyta (*Staurastrum* and *Gloeocystis*) and cyanobacteria (*Microcystis* and *Pseudanabaena*).

After sampling water quality for day 0 measurements, the mesocosms were either left untreated (controls) or treated with a one of seven algaecides (Table 3.2). Mesocosms were randomly assigned. Mesocosms were mixed with a tube sampler for 10 seconds after the application of each product. Integrated water samples were then collected from each mesocosm on days 1, 3, 7, 14, 21, 28, and 35. Chlorophyll and phycocyanin values were measured on all sampling days. Phytoplankton and zooplankton samples were counted for days 0, 1, 7, and 35.

Products were assessed foremost on their ability to reduce cyanobacteria. Changes in the total phytoplankton and zooplankton biomass were also assessed for all product treatments. A repeated-measures analysis of variance (RM-ANOVA) using a restricted maximum log-likelihood (REML) method was used to assess these differences in total phytoplankton (measured as chlorophyll and phytoplankton biovolume), cyanobacterial density (phycocyanin), and zooplankton density between product treatments over time. Tukey's multiple comparison tests were used to compare mean effects among treatments. The analysis was performed using the *nlme* package in R (Pinheiro et al., 2020).

Results

Laboratory experiment

Seven algaecides were tested at four treatment concentrations over the 14-day laboratory experiment by measuring changes in phytoplankton (measured as chlorophyll; Figure 3.2a and c) and cyanobacterial (measured as phycocyanin; Figure 3.2b and d) abundances over time. Briefly, across all products, there were large effects of treatment ($p < 0.000001$), time ($p \leq 0.021$), and the treatment x time interaction ($p < 0.000001$) on both chlorophyll and phycocyanin concentrations (RM-ANOVA). The copper-based products, $CuSO_4$ and Captain, significantly reduced phytoplankton and cyanobacteria with concentrations ≥0.2 mg/L as Cu ($p \leq 0.05$; Appendix Figure C1 and C5). At these concentrations, cyanobacteria were fully removed from the jars with both products by day 3, while total phytoplankton biomass significantly declined and largely remained <200 µg/L (compared to starting chlorophyll concentrations ~500 µg/L) in both copper products for the duration of the trial. H_2O_2-based products, liquid H_2O_2 and granulated PAK-27, both significantly reduced total phytoplankton and cyanobacteria at concentrations ≥5 mg/L as H_2O_2 (Appendix Figure C2 and C6). Although biomass did decrease in the first three days, both total phytoplankton and cyanobacteria again increased over the 14-day trial, but still remained lower than the control. Peracetic acid-based products, Peraclean and VigorOx SP-15, significantly reduced phytoplankton and cyanobacteria with concentrations ≥2 mg/L as volume, with the greatest effects observed at concentrations ≥10 mg/L ($p \leq 0.05$; Appendix Figure C3 and C7). Cyanobacteria remained at or near-to zero after day 1 in concentrations ≥5 mg/L. Phytoplankton increased over the 14-day experiment in concentrations less than ≤5 mg/L and ≤12 mg/L in the Peraclean and VigorOx SP-15 treatments, respectively. However, phytoplankton and cyanobacteria still remained lower than that of the control throughout the entire experiment after treatment. For Phoslock, only the ratio of 50:1 (kg phoslock: kg waterbody phosphorus) reduced phytoplankton abundance when compared to the

control (Appendix Figure C4) while the 200:1 Phoslock treatment was the only treatment to reduce cyanobacteria relative to the control (Appendix Figure C8).

From the various concentrations at which the seven algaecides were tested, we identified the following concentrations to be tested in the field experiment: 0.4 mg/L of $CuSO_4$ and Captain, 10.2 mg/L of liquid H_2O_2 and PAK-27, 10 mg/L of VigorOx SP-15 and Peraclean, and 200:1 ratio for Phoslock (Table 3.2). Across these products, all had at least one concentration that significantly reduced both total phytoplankton (Figure 3.2a and c) and cyanobacteria (Figure 3.2b and d) over the entire 14-day experiment when compared to the control.

Field experiment

A 35-day field mesocosm experiment evaluated seven algal control products on phytoplankton (as chlorophyll and biovolume), cyanobacteria (as phycocyanin), and zooplankton biomass relative to a control. Although some treatments caused large, rapid declines in chlorophyll (starting values averaged ~56 µg/L), all treatments returned to near initial conditions within 21 days (Figure 3.3a and c). Although there were significant effects of treatment ($p = 0.00170$) and time ($p < 0.001$) in the field experiment, treatment x time interaction was not significant ($p = 0.293$) on chlorophyll (RM-ANOVA). Only $CuSO_4$ decreased chlorophyll more than the control across the entire 35 day experiment ($p < 0.05$; Figure 3.3a). In the first seven days, Captain and $CuSO_4$ significantly reduced phytoplankton before increasing overtime (Figure 3.3a and c). Liquid H_2O_2 also reduced chlorophyll, but this reduction was short-lived considering that chlorophyll peaked on day 7 in this treatment (Figure 3.3a and c). Chlorophyll concentrations for liquid H_2O_2 and the controls were statistically similar (Figure 3.3a). Several treatments, including Peraclean, VigorOx SP-15,

Phoslock, and PAK-27, had similar chlorophyll concentrations relative to the control the entire experiment (Figure 3.3a and c).

Initial cyanobacterial concentrations (as phycocyanin) averaged ~15 µg/L at the start of the experiment (Figure 3.3b and d). Although there were significant effects of treatment ($p < 0.00001$) and a treatment x time interaction ($p < 0.00001$) in the field experiment, time was not significant ($p = 0.425$) on phycocyanin (RM-ANOVA). All products reduced cyanobacterial densities after 1 day except for Phoslock. Interestingly, liquid H_2O_2 led to increased cyanobacteria relative to the control on day 7 (Figure 3.3b and d). In total, all products except for liquid H_2O_2 had a significantly lower cyanobacterial concentration than that of the control during the 35-day experiment ($p \leq 0.05$, Figure 3.3b and d).

Phytoplankton biovolume

Phytoplankton biovolume was estimated for all mesocosms for days 0, 1, 7, and 35 of the field experiment. Average starting phytoplankton biovolume averaged ~1.17×10^7 μm^3/mL across all products (Figure 3.4a). Chlorophytes were the dominant phytoplankton, averaging 9.89×10^6 μm^3/mL (55.9% of starting biovolume) across all enclosures. Cyanobacteria had the second greatest density in the mesocosms, averaging 1.66×10^6 μm^3/mL (9.4% of starting biovolume) between all enclosures (Figure 3.5a). Additional phytoplankton groups observed included cryptophytes, dinoflagellates, euglenoids, and diatoms, but the presence of these taxon were generally not substantial (Figure 3.5a).

Across all products during the 35-day experiment, there were significant effects of treatment ($p < 0.000001$), time ($p = 0.0448$), and treatment x time interaction ($p = 0.0022$) on phytoplankton biovolume (RM-ANOVA). All products, except Phoslock, reduced phytoplankton biovolume during the experiment first day when compared to that of the

control, however phytoplankton rebounded to initial concentrations over the duration of the experiment (Figure 3.4a). Phytoplankton biovolume in the two copper-based treatments (Captain and $CuSO_4$) were the only products to remain significantly lower to that of the control across the 35-day experiment ($p \leq 0.05$; Figure 3.4a). The final ratio of cyanobacteria to total phytoplankton varied greatly between product treatments with Captain, $CuSO_4$, and H_2O_2 having ≥50% of their total biovolume comprising of cyanobacteria (Figure 3.5a). Although some variation in findings did occur, phytoplankton biovolume generally mirrored the trends observed in the algal pigment data (Figure 3.3).

Zooplankton dry biomass

Zooplankton biomass was estimated for all mesocosms on days 0, 1, 7, and 35 of the field experiment. The average starting zooplankton dry biomass was ~602 µg/L across all treatments (Figure 3.4b). Mesocosms contained a mixture of cladoceran and copepod taxa, contributing 38% and 62% of the total biomass, respectively, at the start of the experiment. Starting densities of these genera varied. On average, mesocosms contained *Ceriodaphnia* (1% of total starting biomass), *Diaphanosoma* (10%), Bosmina (25%), copepod nauplii (15%), calanoid copepods (46%), and cyclopoid copepods (4%).

There were large effects of treatment ($p < 0.000001$), time ($p < 0.000001$), and treatment x time interaction ($p < 0.000001$) on zooplankton dry biomass (RM-ANOVA) during the 35-day experiment. Only $CuSO_4$, Captain, and PAK-27 treatments were significantly lower than the control for zooplankton biomass ($p \leq 0.05$; Figure 3.4b) while biomas in the four other products, although varying in value over time, did not significantly differ from the control. $CuSO_4$ zooplankton biomass remained the lowest over the 35 days. Interestingly, liquid H_2O_2 contained the lowest zooplankton biomass of any product after day 1, but steadily rebounded in number

over the next 35 days. Final (day 35) relative biomass between zooplankton groups were *Ceriodaphnia* (2% of final biomass), *Diaphanosoma* (47%), *Bosmina* (3%), copepod nauplii (3%), calanoid copepods (44%), and cyclopoid copepods (0.4%), although diversity and abundance in biomass varied between products (Fig 3.5b).

Discussion

In this study, we used both a short, microcosm laboratory and five-week, field mesocosm experiment to evaluate seven algal control products in an aquaculture pond. In doing so, both the short- and long-term effectiveness of each product was assessed. The effects of each product on algal pigments (representing phytoplankton and cyanobacteria), phytoplankton biovolume, and zooplankton biomass will be described in the following sections. The focus of this discussion will be given to the findings of the field experiment. As the chlorophyll pigment and total phytoplankton biovolume data are both assessments of total phytoplankton densities in the field experiment, the results of these two assessments with be described within a single section.

Effects on phytoplankton (using chlorophyll and phytoplankton biovolume data)

Phytoplankton communities in the mesocosms at the start of the experiment were dominated by green algae (Figure 3.5). Cyanobacteria were the next largest taxon present. Of the products tested, Captain and $CuSO_4$ best reduced phytoplankton abundance in the field experiment (Figure 3.3 and 3.4). When assessing the chlorophyll data, both Captain and $CuSO_4$ significantly reduced chlorophyll within the first 7 days of the experiment, and $CuSO_4$ was the only product to have significantly lower chlorophyll levels to that of the control for the duration of the 35-days (Figure 3.3). Similarly, phytoplankton biovolume data in Captain and $CuSO_4$ treatments were significantly lower than the control (Figure 3.4).

The broad-spectrum toxicity and extended duration of select copper products have been observed in prior studies (Murray-Gulde, 2002; Viriyatum and Boyd, 2016). The efficiency of copper does vary and can often be attributed to the form in which it is applied. For instance, Viriyatum and Boyd (2016) observed that a single treatment of $CuSO_4$ encapsulated in a slow-release coating had an equally comparable reduction in phytoplankton over four months when compared to ponds treated with granular $CuSO_4$ applied weekly. Although differences between Captain and $CuSO_4$ were observed in this study, both products were found to be the most efficient at reducing phytoplankton over time (when considering chlorophyll and algal biovolume data).

VigorOx SP-15 and Peraclean reduced phytoplankton similar to that of the copper-based products in the laboratory experiment (Figure 3.2) but caused negligible effects on phytoplankton in the field (Figure 3.3 and 3.4). Indeed, it was observed in the field experiment that phytoplankton with both products increased from day 0 to 1 (Figure 3.3 and 3.4). This significant difference between the laboratory and field studies is likely due to contact time, species assemblages, and more ideal conditions in the laboratory. In addition, such discrepancies between lab and field-based studies may indicate how short-term, laboratory studies might poorly reflect the effectiveness of algaecides in nature. Yet, VigorOx SP-15 and Peraclean did reduce cyanobacteria while having small effects on other algae in the field, including beneficial green algae. Such findings would benefit farmers as they seek to balance the presence of algae to support dissolved nutrient removal and promote oxygenation within ponds while selecting against cyanobacteria.

Granulated PAK-27 and liquid H_2O_2 produced similar reductions of phytoplankton in the laboratory and field study. However, unlike PAK-27, liquid H_2O_2 produced an immediate

decline in phytoplankton that quickly rebounded to values greater than that of the control in the following days and weeks. Interestingly, only the granulated H_2O_2-based product selectively reduced cyanobacteria. The effectiveness of H_2O_2 as an algaecide has been noted to vary among cyanobacterial species and phytoplankton taxon for both PAK-27 (Sinha et al., 2018) and liquid H_2O_2 (Yang et al., 2018). Such selectiveness may be of use to resource managers wishing to keep some amount of algae present within their system.

Phoslock did not significantly reduce phytoplankton relative to the control in the laboratory or field experiment. Phoslock targets phosphorus by binding and removing it to the sediments (Bishop et al., 2018). The efficiency of this product is meant for the long-term control of phosphorus in systems leading to the eventual change in nutrient ratios and thereby a reduction in phytoplankton density. This is likely the reason for its undetectable effect in the short-term in the laboratory experiment as well as small effects in the field experiment. The constant addition of nutrients to the water column by way of feed and fish waste-products may further reduce the success of Phoslock in intensive aquaculture. However, the effect of Phoslock on removing cyanobacteria showed promise in this study (to be discussed).

Effects on cyanobacterial biomass

Captain and $CuSO_4$ both effectively reduced cyanobacteria in the laboratory and field experiments (Figure 3.2 and 3.3) reflecting the results documented in prior studies (Murray-Gulde, 2002; Viriyatum and Boyd, 2016). Although a concentration of 0.4 mg/L as copper was used in this study, others have used smaller, repeated doses to remove cyanobacterial genera capable of producing off-flavors in farm ponds (Schrader et al., 2005). Moreover, treatments comparable to that used in this study have been shown to reduce cyanobacterial genera capable of producing microcystin (Greenfield, 2014). Off-flavors and microcystin were too low to be

detectable in the collected water samples of this study, and therefore not reported. Kansole and Lin (2017) found that hydrogen peroxide (20 mg/L) could degrade microcystin compounds while $CuSO_4$ (2 mg/L) could not, and both treatments had a deleterious effect on bacterial populations that could degrade microcystin naturally. Such reports reflect copper's ability to reduce phytoplankton, but not cyanotoxins at environmentally relevant concentrations. It was observed that Captain and $CuSO_4$ enclosures both were both dominated by cyanobacteria by the end of the 35-day field experiment (Appendix Figure C5). Although phytoplankton in Captain and $CuSO_4$ treatments were the lowest observed across the tested products, such a shift in the dominant phytoplankton taxa could promote cyanobacterial blooms in the future.

Similar to that of the copper-based algaecides, VigorOx SP-15 and Peraclean (peracetic acid-based) significantly reduced cyanobacteria in both the laboratory and field experiments. Yet, both products did not significantly reduce phytoplankton in the field experiment, which were dominated by green algae (Figure 3.2 and 3.3). This selective effectiveness has been observed for other algaecides, such as H_2O_2 (Yang et al., 2018), which is a chemical also present in VigorOx SP-15 and Peraclean. Reasons for this selectiveness may be attributed to the lack of a cell wall in prokaryotes (e.g., cyanobacteria; Yang et al., 2018), the proximity of the photosynthetic apparatuses to the plasma membrane (Yang et al., 2018), or the overall ability to degrade bacterial cell membranes (Mikula et al., 2012). Once hydrogen peroxide enters into the cell of cyanobacteria, it induces oxidative stress, damaging proteins, genes, and photosystems (Liu et al., 2005; Latifi et al., 2008), and can be compounded by UV light exposure (Drábková et al., 2012) and/or the presence of iron (Zepp et al., 1992). The selective effect of H_2O_2 against cyanobacteria was observed in the field experiment for most treatments, except liquid H_2O_2.

However the selectivity of peracetic acid among phytoplankton taxa is understudied and should be further researched.

Liquid H_2O_2 and PAK-27 yielded similar reductions in cyanobacterial densities in the laboratory experiment (Figure 3.2 and 3.5). However, substantial differences were observed between both the findings of laboratory and field experiments as well as between the two products in the field (Figure 3.2, 3.3, and 3.5b). It was observed in the field experiment that liquid H_2O_2 first reduced cyanobacteria, but phycocyanin then increased greater than the control. In contrast, granulated H_2O_2 kept phycocyanin densities well below that of the control for the duration of the experiment (Figure 3.3b and d). Such differences again reflect the dissonance between laboratory and field studies. It should be noted in the field experiment that both liquid H_2O_2 and PAK-27 reduced cyanobacteria for the first three days of the experiment. This finding may support that H_2O_2-based products are effective at quickly removing toxic and problematic cyanobacterial species, as has been suggested in prior studies (Barrington et al., 2013; Sinha et al., 2018; Yang et al., 2018), but repeated treatments may be required for the continual suppression of a bloom (as suggested by Barrington et al., 2013). Prior research has also observed that hydrogen peroxide may degrade cyanotoxins, negating their negative effects once released from the cells of cyanobacteria (Barrington et al., 2013; Kansole and Lin, 2017); however, concentrations needed to achieve this are relatively high (e.g., 20 mg/L; Kansole and Lin, 2017) and may not be economically feasible for fish farmers to utilize (to be discussed).

Similar to the H_2O_2- and peracetic acid-based products, Phoslock was also found to have a significant effect on cyanobacteria in the field experiment, but not on phytoplankton in general (Figure 3.3). Such a reduction was likely due to the removal of phosphorus out of the water column as the decrease of cyanobacteria was gradual in the field experiment (Van Oosterhout

and Lürling, 2013). However, in the laboratory experiment, the removal of cyanobacteria was much more rapid and did not have a similar effect on other phytoplankton taxa (Figure 3.2). This finding may suggest that Phoslock bound and removed cyanobacteria upon its application into the jars and that its removal is taxon-specific. Phoslock and other clay compounds have been shown to bind directly with phytoplankton (including cyanobacteria) and remove them from the water column (Pan et al., 2011; Van Oosterhout and Lürling, 2013). The selectivity of such clays on their possible selectivity against cyanobacteria is understudied and should be researched further.

Effects on zooplankton biomass

The seven algal control products revealed varying effects on zooplankton biomass during the field experiment (Figure 3.4b). Although zooplankton biomass was reduced by most treatments relative to the controls in the first 7 days, zooplankton returned to values similar to that of the control in the Phoslock, liquid H_2O_2, Peraclean, and VigorOx SP-15 treatments (Figure 3.4b). In contrast, $CuSO_4$, Captain, and PAK-27 each significantly reduced zooplankton densities below that of the control over the 35 days. In contrast, copper-based products had the greatest, sustained negative effect on zooplankton biomass (Figure 3.4b). Significant zooplankton reductions after a treatment of copper-based algaecides have been observed in prior studies. McIntosh and Kevern (1974) reported that treatments of 3 mg/L of $CuSO_4$-$5H_2O$ significantly reduced copepods and cladocerans in field treatments. However, it has also been observed that water quality factors such as dissolved organic matter may “buffer” the toxicity of copper to zooplankton (De Schamphelaere et al., 2004). These factors may influence the effect when copper is applied to cyanobacterial blooms in more productive systems than that used in this study, although such variables were not measured in our field experiment. Further the

internal loading of copper onto a aquaculture was also not assessed in this experiment. Such factors has been observed in the sediments aquaculture ponds (Han et al., 2001), and may have additional adverse effects to nontarget organisms and phytoplankton.

VigorOx SP-15 and Peraclean had minimal effects on zooplankton biomass in this study. As with copper-based products, the toxicity of peracetic acid to zooplankton has been found to be dependent on water quality variables (e.g., dissolved organic matter, salt; Liu et al., 2015). Interestingly, Liu et al. (2015) found that the toxicity of peracetic acid products to zooplankton will increase with the amount of H_2O_2 that a product also contains. Of the H_2O_2-based products used in this study, PAK-27 also significantly reduced zooplankton biomass, and liquid H_2O_2 greatly reduced biomass after the initial treatment by day 1, but the densities in the liquid H_2O_2 treatment returned to densities not significantly to the of the control by the end of the 35-day experiment. The toxicity of H_2O_2 to zooplankton has been assessed on numerous occasions (Barrington et al., 2013; Reichwaldt et al., 2012; Yang et al., 2018), and findings of these past studies are aligned with the results from our field experiment.

Lastly, the effect of Phoslock on zooplankton biomass was minimal and not significantly different to that of the control. Lürling and Tolman (2010) observed that the active ingredient (lanthanum) of Phoslock was not toxic to *Daphnia* at concentrations up to 1000 µg/L. It is likely that the rapid removal of Phoslock out of the water column or limited toxicity reduced its effectiveness on the zooplankton biomass in this study.

Costs per product treatment

The average costs to treat a 20 acre-foot pond were calculated based on an example dosage of each product used in this study as well as prices for these products as of April 2020 (Table 3.3). $CuSO_4$ had a remarkably lower cost and application volume than any other

product. This relatively low price likely reflects the wide availability and popularity of $CuSO_4$, and the relatively lower application volume contributes to copper's ease of use for fish farmers. Conversely, PAK-27 had the highest cost. It should be noted that all costs are subject to change and may be lower if a product is purchased at a larger quantity. Further, prices may be influenced if an algaecide gains USEPA approval for use in food-fish aquaculture. At this time, $CuSO_4$ is the only product fully allowed by the EPA. However, PAK-27, Captain, and Phoslock are approved to control nuisance algae and cyanobacterial blooms in some states.

Disclaimers

An algaecide must first receive USEPA approval before its use in food fish aquaculture in the U.S., requiring significant effort and costs. It should be noted that some algaecides are approved for use to combat nuisance plants and algae in non-aquaculture ponds. Such approvals vary from state to state. In general, "any product or device that is used or implied to control algae (including cyanobacteria) must be registered by the USEPA under FIFRA" **(**Laughinghouse et al. 2020). Moreover, guidelines and directions provided by the vendor on the labeled instructions should be explicitly followed. The objectives of these experiments were to compare efficacy in a demonstration/research environment and not to endorse the use of any specific product. Local, state and federal authorities should be consulted before any chemical is applied to surface waters.

The assessment of oxygen during the night hours or amounts levels of ammonia were not checked in within this study. Such factors can be major issues to fish after major a phytoplankton or plant die-off as oxygen concentrations will be depleted through microbial disposition (Chislock et al., 2013a), and ammonia concentrations will increase through the breakdown of organic material (Farnsworth-Lee and Bake, 2000) or through the lack of uptake by

phytoplankton (Boyd et al., 1975). Moreover, both off-flavors and microcystin can be released from cyanobacteria as their cells rupture, an issue that can be spurred on by algaecide applications (Jones and Orr, 1994; Jüttner and Watson, 2007). Applicators should monitor their ponds for these parameters after an application of algaecide to avoid serious issues.

Conclusions

This study utilized both a laboratory and field study to compare algal control products to one routinely used ($CuSO_4$) in farm-pond aquaculture as well as a treatment-less control. Our findings indicate that copper-based products, Captain and $CuSO_4$, had the greatest reduction of phytoplankton and cyanobacteria in both the laboratory and field studies. $CuSO_4$ also had the lowest treatment costs relative to the other algaecides tested. However, it was observed that copper-based products had significant adverse effects on zooplankton densities and its broad-spectrum toxicity may not be useful in all situations.

Peracetic-acid based products, VigorOx SP-15 and Peraclean, as well as a granulated H_2O_2-based product (PAK-27), significantly removed cyanobacteria while having small effects on other phytoplankton, specifically beneficial green algae, during the field experiment. Moreover, peracetic acid-based products had small effects on zooplankton when compared to the control treatment. Surprisingly, liquid H_2O_2 showed to have short-lasting effects on phytoplankton abundance while also promoting cyanobacteria by the end of the field experiment. In addition, large negative effects of both H_2O_2-based products on zooplankton was observed. The cost of the peracetic acid- and H_2O_2-based products ranged from moderate-to-high relative to the others tested.

The clay product, Phoslock, showed little significant effect on phytoplankton in the field experiment, but significantly reduced cyanobacterial abundance. Given that the mechanism that

Phoslock exploits to control phytoplankton is by binding phosphorus and making it unavailable for phytoplankton, it may take some time for this treatment to show effects relative to true algaecides tested in this study. In the laboratory experiment, cyanobacterial densities were immediately reduced upon the application of Phoslock and may indicate its ability to bind and selectively remove cyanobacteria from the water column. The cost of Phoslock was the second highest treatment used in this study, but perhaps may be circumvented if fewer applications are needed.

In this study, it was made clear that extended results from the tightly controlled lab studies to the field should be done with caution. Also, the effects of most algaecides on phytoplankton is short-lived. As this study was performed in floating mesocosms, we encourage the use of full scale pond trials to rigorously test the multiple algaecides under uniform conditions to evaluate their efficacy. Aspects such as mixing, sedimentation, and application methods may have an effect on treatment effectiveness and longevity.

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Table and Figure legends

Table 3.1 Algaecides used for laboratory experimentation, including dosage.

Table 3.2 Product dosage used in the field experiment.

Table 3.3 Treatment costs of products to treat 20 acre ft. Prices were determined for an example dosage and the cost of a container of a product that is commonly available for fish farms to purchase in the southeastern USA as of April 2020.

Figure 3.1 Floating dock that held the mesocosms for the field experiment. Additional mesocosms are pictured here that were not used as part of this experiment.

Figure 3.2 Dynamics of phytoplankton (as chlorophyll [µg/L]) or cyanobacteria (as phycocyanin [µg/L]) across a 14-day laboratory, microcosm (0.4 L) experiment where seven algaecides were tested relative to an algaecide-less control (0.0 mg/L). Only data for the targeted concentration used in the field experiment for each algaecides are shown. Data for other algaecides concentrations are available in Appendix C. The Phoslock application rate was calculated as 200 units (µg) of Phoslock for every unit (µg) of total phosphorus in a waterbody given an estimated volume. Panels A and B show absolute data, while panels C and D show relative concentrations (calculated as [product treatment mean – control mean]/control) mean for each sampling day. Error bars in panels A and B represent one standard error. Letters in brackets after each product are results from Tukey's multiple comparison tests. Products sharing the same letter are not statistically different ($p \geq 0.05$) over the entire 35 days using repeated measures ANOVA.

Figure 3.3 Dynamics of phytoplankton (as chlorophyll [µg/L]) or cyanobacteria (as phycocyanin [µg/L]) across a 35-day field mesocosm (1,310 L) experiment where seven algaecides were tested relative to an algaecides -less control (0.0 mg/L). The Phoslock application rate was calculated as 200 units (µg) of Phoslock for every unit (µg) of total phosphorus in a waterbody given an estimated volume. Panels A and B show absolute data, while panels C and D show

relative concentrations (calculated as [product treatment mean – control mean]/control mean) for each sampling day. Error bars in panels A and B represent one standard error. Letters in brackets after each product are results from Tukey's multiple comparison tests. Products sharing the same letter are not statistically ($p \geq 0.05$) different over the entire 35-day experiment using repeated measures ANOVA

Figure 3.4 Dynamics of (A) phytoplankton biovolume (µm3/ml) and (B) zooplankton dry biomass (µg/L) across a 35-day field mesocosm (1,310 L) experiment where seven algaecides were tested relative to an algaecide-less control (0.0 mg/L). The Phoslock application rate was calculated as 200 units (µg) of Phoslock for every unit (µg) of total phosphorus in a waterbody given an estimated volume. Error bars in panels A and B represent one standard error. Letters in brackets after each product are results from Tukey's multiple comparison tests using log10-transformed data. Products sharing the same letter are not statistically ($p \geq 0.05$) different over the 35 day experiment using repeated measures ANOVA.

Figure 3.5 Trends in (A) phytoplankton and (B) zooplankton community structure across four sampling days (0 (pre-treatment), 1, 7, and 35) of a 35-day field mesocosm (1,310 L) experiment where seven algacides were tested relative to an algaecide-less control (0.0 mg/L or no Phoslock added). The Phoslock application rate was calculated as 200 units (µg) of Phoslock for every unit (µg) of total phosphorus in a waterbody given an estimated volume.

Table 3.1

Product	Active ingredient	Dosage	Brand	Reference treatment concentration
Copper sulfate	copper	0.2, 0.4, 0.6, 1 mg/L as copper	VWR	Viriyatum and Boyd 2016
Captain (chelated copper)	copper	0.2, 0.4, 0.6, 1 mg/L as copper	SePRO	Viriyatum and Boyd 2016
Hydrogen peroxide, liquid	hydrogen peroxide	2, 5, 10.2, 12 mg/L as hydrogen peroxide	VWR	Yang et al. 2018
PAK®-27 (sodium carbonate peroxyhydrate)	hydrogen peroxide	2, 5, 10.2, 12 mg/L as hydrogen peroxide	SePRO	Sinha et al. 2018
VigorOx SP-15 ®	peracetic acid	2, 5, 10, and 12 mg/L total volume	Peroxychem	Enviro Tech Chemicals 2003
Peraclean ®	peracetic acid	2, 5, 10, and 12 mg/L total volume	Evonik	Enviro Tech Chemicals 2003
Phoslock ®	quartz and titanium dioxide	200:1, 100:1, 50:1, 25:1 ratios of Phoslock to 1 kg of phosphorous	SePRO	Bishop and Richardson 2018

Table 3.2

Chemical	Dosage	Form added
Copper sulfate	0.4 mg/L as copper	Dissolved in liquid
Captain ®	0.4 mg/L as copper	Diluted liquid
Hydrogen peroxide, liquid	10.2 mg/L as hydrogen peroxide	Diluted liquid
PAK-27 ® (sodium carbonate peroxyhydrate)	10.2 mg/L as hydrogen peroxide	Dissolved in liquid
VigorOx SP-15 ®	10 mg/L total volume	Diluted liquid
Peraclean ®	10 mg/L total volume	Diluted liquid
Phoslock ®	200:1 ratio of kg Phoslock to 1 kg of ambient phosphorus in a lake	Slurry

Table 3.3

Product	Cost per container ($)	Container quantity	Dose	Cost to treat 20 acre ft ($)
Hydrogen peroxide, liquid	1250.00	1041 L	10.0 (mg/L)	897.65
PAK-27 ®	60.00	23 kg	10.0 (mg/L)	2417.21
Peraclean ®	74.40	19 L	10.0 (mg/L)	843.15
VigorOx SP-15 ®	5580.00	1249 L	10.0 (mg/L)	975.19
$CuSO_4$	58.99	23 kg	0.4 (mg/L)	64.49
Captain ®	70.00	9 L	0.4 (mg/L)	671.63
Phoslock ®	88.00	25 kg	200:1 (lake TP of 100 µg/L)	1740.39

Figure 3.1

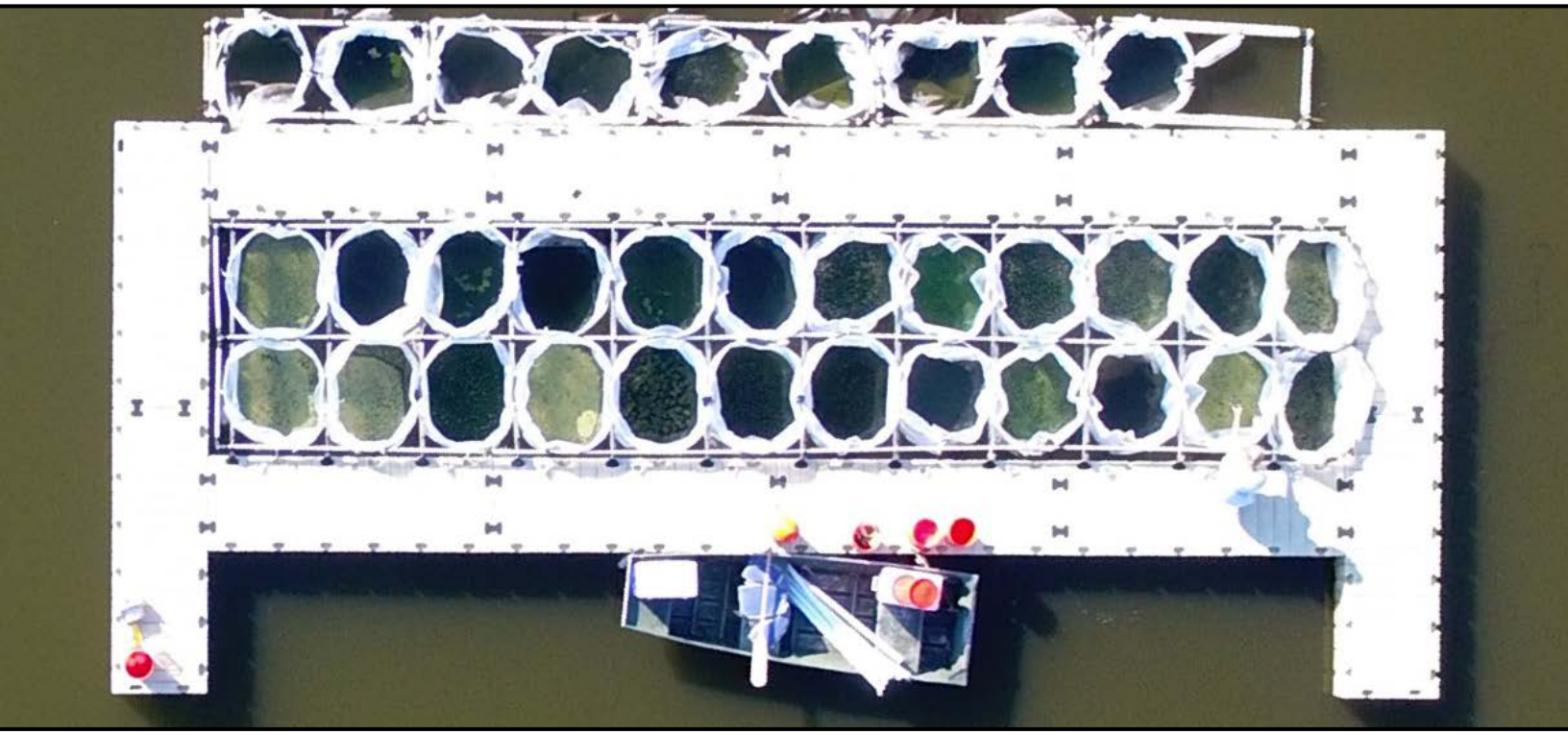


Figure 3.2

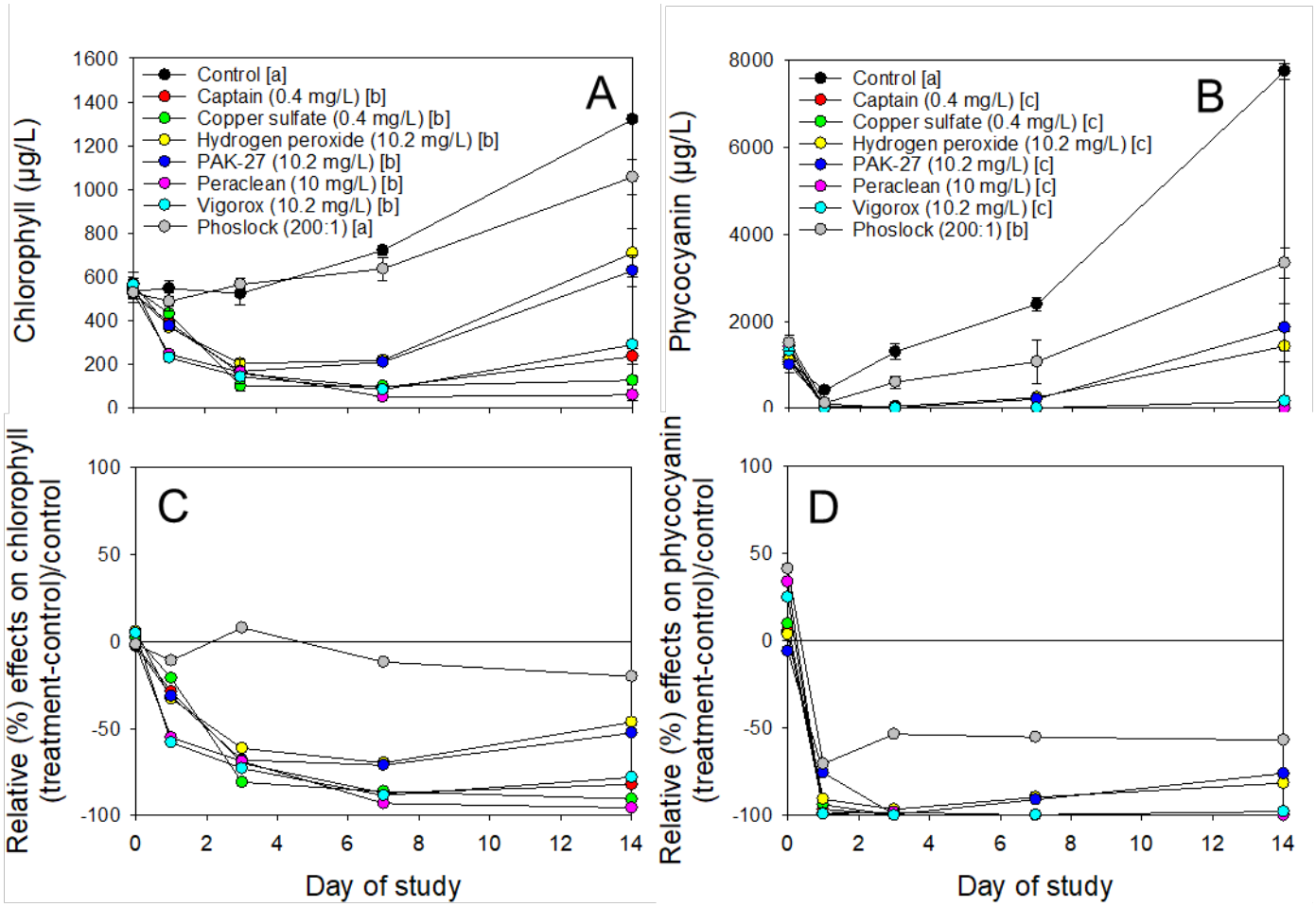

A
Chlorophyll (µg/L)
Control [a]
Captain (0.4 mg/L) [b]
Copper sulfate (0.4 mg/L) [b]
Hydrogen peroxide (10.2 mg/L) [b]
PAK-27 (10.2 mg/L) [b]
Peraclean (10 mg/L) [b]
Vigorox (10.2 mg/L) [b]
Phoslock (200:1) [a]
B
Phycocyanin (µg/L)
Control [a]
Captain (0.4 mg/L) [c]
Copper sulfate (0.4 mg/L) [c]
Hydrogen peroxide (10.2 mg/L) [c]
PAK-27 (10.2 mg/L) [c]
Peraclean (10 mg/L) [c]
Vigorox (10.2 mg/L) [c]
Phoslock (200:1) [b]
C
Relative (%) effects on chlorophyll (treatment-control)/control
Day of study
D
Relative (%) effects on phycocyanin (treatment-control)/control
Day of study

Figure 3.3

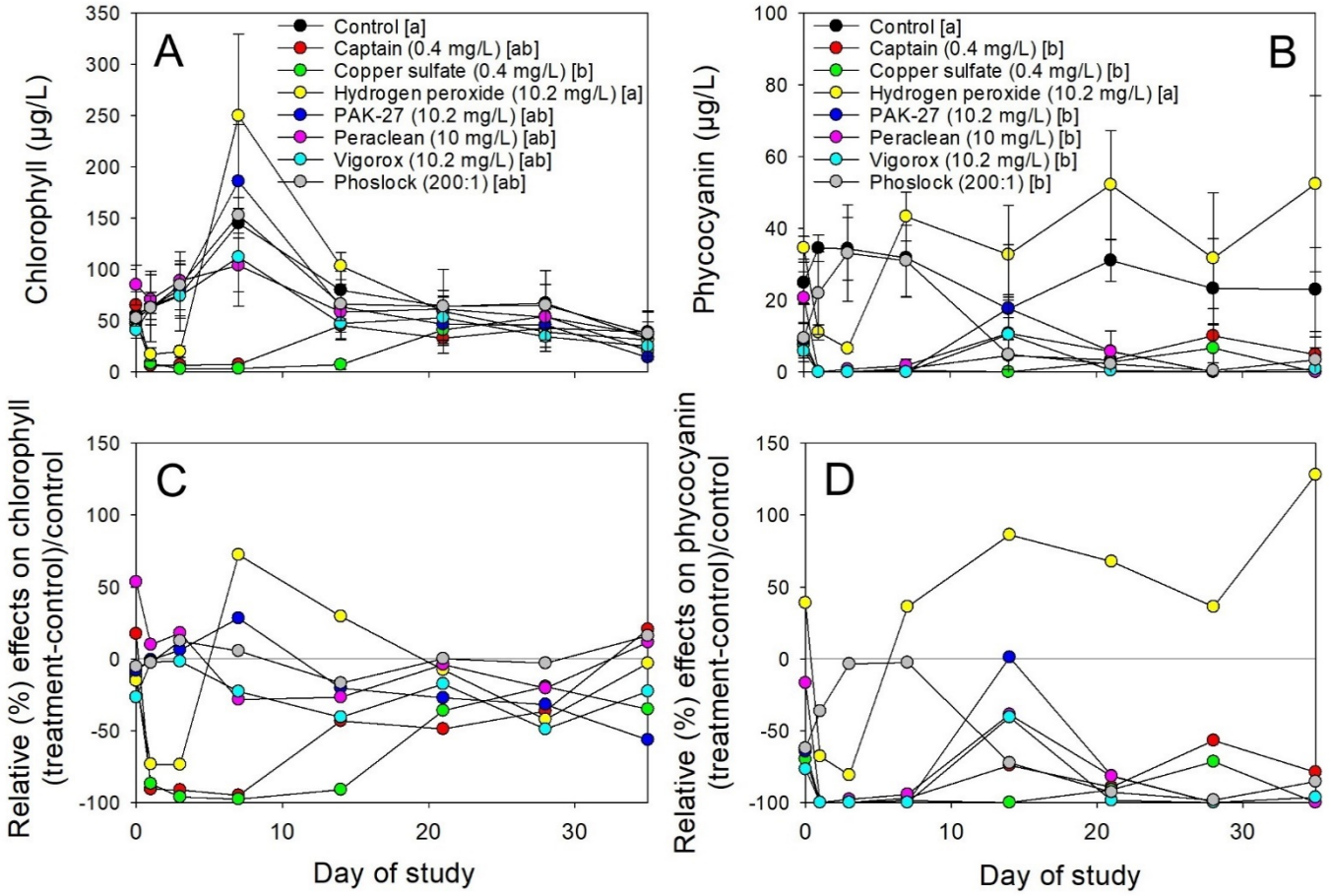

A
Chlorophyll (µg/L)
Control [a]
Captain (0.4 mg/L) [ab]
Copper sulfate (0.4 mg/L) [b]
Hydrogen peroxide (10.2 mg/L) [a]
PAK-27 (10.2 mg/L) [ab]
Peraclean (10 mg/L) [ab]
Vigorox (10.2 mg/L) [ab]
Phoslock (200:1) [ab]
B
Phycocyanin (µg/L)
Control [a]
Captain (0.4 mg/L) [b]
Copper sulfate (0.4 mg/L) [b]
Hydrogen peroxide (10.2 mg/L) [a]
PAK-27 (10.2 mg/L) [b]
Peraclean (10 mg/L) [b]
Vigorox (10.2 mg/L) [b]
Phoslock (200:1) [b]
C
Relative (%) effects on chlorophyll (treatment-control)/control
Day of study
D
Relative (%) effects on phycocyanin (treatment-control)/control
Day of study

Figure 3.4

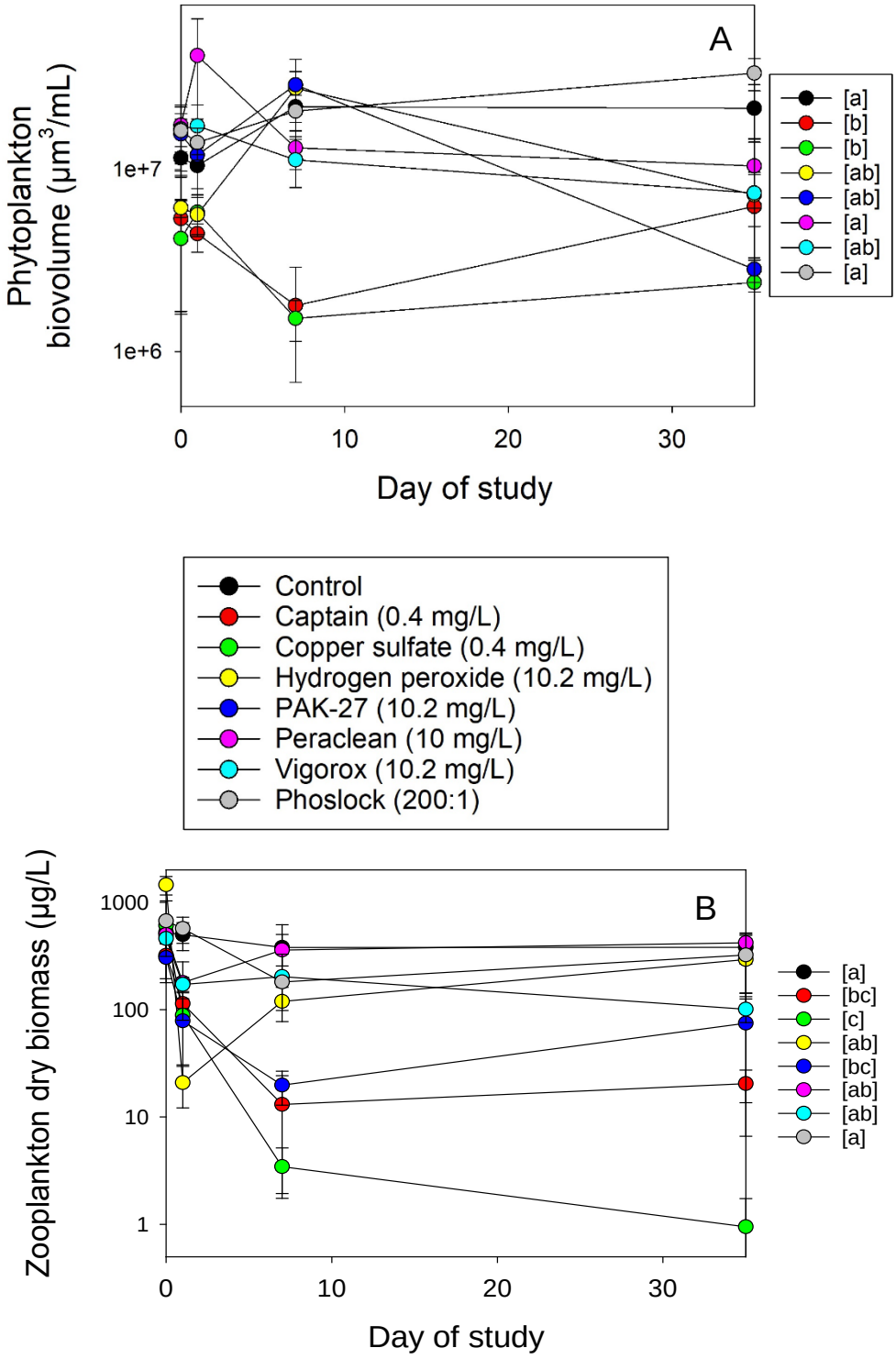
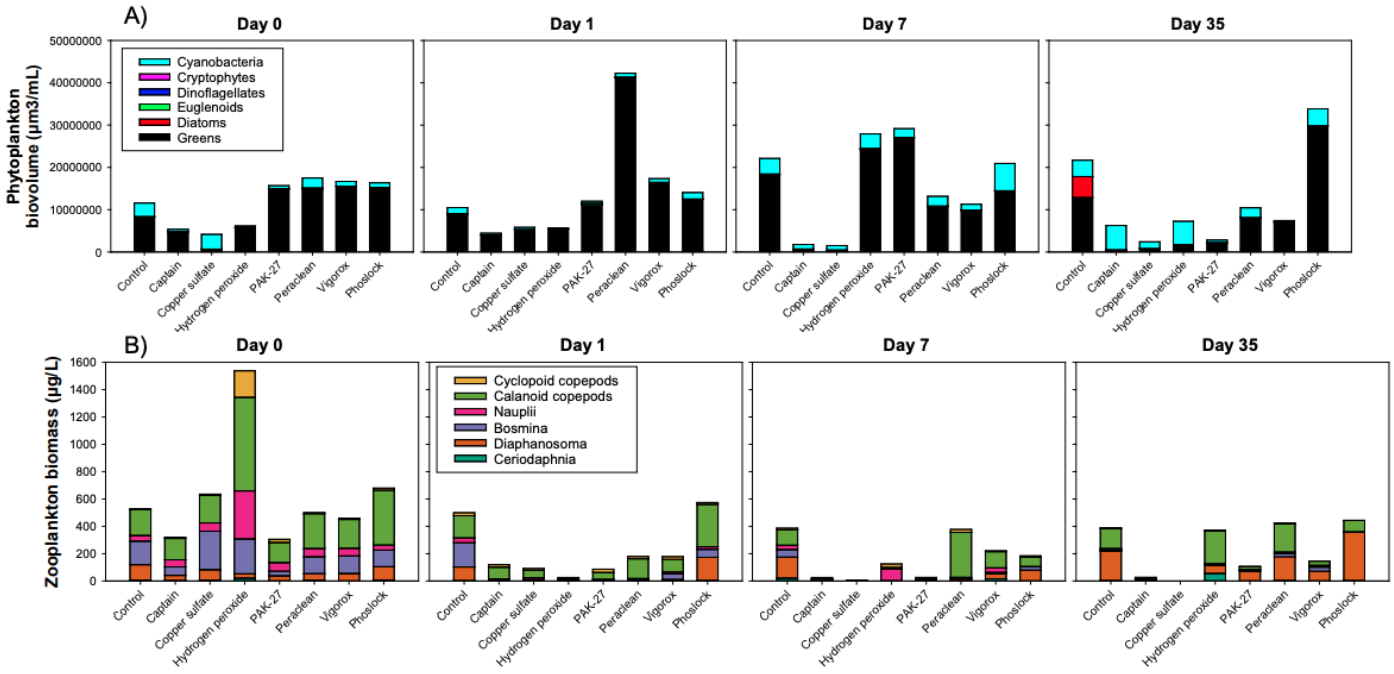

A
Phytoplankton biovolume (µm³/mL)
1e+7
1e+6
0
10
20
30
Day of study
[a]
[b]
[b]
[ab]
[ab]
[a]
[ab]
[a]
Control
Captain (0.4 mg/L)
Copper sulfate (0.4 mg/L)
Hydrogen peroxide (10.2 mg/L)
PAK-27 (10.2 mg/L)
Peraclean (10 mg/L)
Vigorox (10.2 mg/L)
Phoslock (200:1)
B
Zooplankton dry biomass (µg/L)
1000
100
10
1
0
10
20
30
Day of study
[a]
[bc]
[c]
[ab]
[bc]
[ab]
[ab]
[a]

Figure 3.5


A)
Day 0
Day 1
Day 7
Day 35
Phytoplankton biovolume (µm3/mL)
50000000
40000000
30000000
20000000
10000000
0
Cyanobacteria
Cryptophytes
Dinoflagellates
Euglenoids
Diatoms
Greens
Control
Captain
Copper sulfate
Hydrogen peroxide
PAK-27
Peraclean
Vigorox
Phoslock
B)
Zooplankton biomass (µg/L)
1600
1400
1200
1000
800
600
400
200
0
Cyclopoid copepods
Calanoid copepods
Nauplii
Bosmina
Diaphanosoma
Ceriodaphnia

Chapter 4

Complex effects of dissolved organic matter, temperature, and initial bloom density on the efficacy of hydrogen peroxide to control cyanobacteria

Abstract

Harmful cyanobacterial blooms plague reservoirs and lakes used for anthropogenic purposes. Chemical controls are frequently used to mitigate the occurrence of cyanobacterial blooms, as many are fast-acting and effective at reducing cyanobacterial abundance. Recent research has identified hydrogen peroxide (H_2O_2) as an environmentally-friendly alternative to algaecides that have typically been used, such as copper sulfate. Such research has assessed the effectiveness of H_2O_2 on cyanobacteria and phytoplankton under laboratory and field conditions, finding that factors such as ultraviolet (UV) light intensity and the iron content of water greatly influence its efficacy to reduce cell densities. To build on past studies, this study seeks to further understand how well H_2O_2 treatments reduce cyanobacteria in complex eutrophic conditions. We assessed the effectiveness of H_2O_2 (at treatments of 0 - 16 mg L^{-1}) under varying environmental conditions, such as (1) dissolved organic matter concentrations (humic acid; 0 - 60 mg L^{-1}), (2) temperatures (20, 25, and 32 °C), and (3) initial algal biomass, under a controlled laboratory setting. Neither variation in dissolved organic matter concentration or temperature impacted the effectiveness of H_2O_2 at reducing cyanobacteria. However, initial algal biomass as well as H_2O_2 treatment greatly influenced the effectiveness of the algaecide. Across all experiments, H_2O_2 concentrations of 0.03 - 0.12 mg H_2O_2 L^{-1}/µg chlorophyll L^{-1} were effective at significantly reducing cyanobacteria. Thus, water resource managers are encouraged to consider how ambient levels of algal biomass may affect the ability of H_2O_2 to control algal blooms prior to treatment.

Introduction

The overabundance of cyanobacteria in aquatic ecosystems is an issue of increasing concern in areas such as aquaculture (Schrader et al., 2016), drinking water (Kehoe et al., 2015), wastewater (Barrington et al., 2008), and recreation (Francy et al., 2016). Both algal and cyanobacterial blooms can cause hypoxic conditions through microbial degradation of decaying algal material, while select species of cyanobacteria can produce secondary metabolites toxic to fish, humans, and other organisms (e.g., microcystins, nodularins), or that will affect the taste and odor of drinking water or fish fillets (e.g., geosmin, 2-methylisoborneol; Kehoe et al., 2015; Schrader et al., 2016). Such issues cause significant economic loss to numerous industries that rely on quality water for their operations and has encouraged research focused on methods and approaches to combat cyanobacterial blooms when they occur.

Chemical controls are frequently used to mitigate the occurrence of cyanobacterial and algal blooms, as algaecides are effective and fast-acting at reducing phytoplankton (Buley et al., 2021). Recent research has identified hydrogen peroxide (H_2O_2) as a possible alternative to algaecides more routinely used, such as copper sulfate (Yang et al., 2018; Buley et al., 2021). Hydrogen peroxide kills phytoplankton through the inhibition of photosystem I and II, the electron transport chain (Greenfield, 2014), and/or through the peroxidation of the lipid membrane (Wang et al., 2012). Hydrogen peroxide has also shown other benefits related to algal blooms, namely that it can degrade cyanobacterial toxins, such as microcystin (Barrington et al., 2013). Additional benefits of H_2O_2 include breaking down into water and oxygen, leaving behind no chemical residuals, and greater selectivity for cyanobacteria over beneficial, non-toxic green algae, as cyanobacteria lack rigid cell walls (Yang et al., 2018). Such selectivity is beneficial for

industries, such as aquaculture, that seek to reduce cyanobacteria because of their toxin and off-flavor production, but need phytoplankton to facilitate oxygen production and ammonia uptake.

The influence of water-quality conditions on the efficacy of H_2O_2 has started to be addressed under laboratory (Drábková et al., 2007), field mesocosm (Yang et al., 2018) and whole-lake studies (Matthijs et al., 2012). As a reactive oxygen species (ROS), H_2O_2 readily reacts with organic substances and metals (Zepp et al., 1992; Crafton et al., 2019), therefore negatively affecting its efficiency as an algaecide when exposed to these conditions. It has also been observed that the species of cyanobacteria present in blooms influences the effectiveness of H_2O_2 treatments, perhaps due to morphological differences or the presence/absence of extracellular polymeric substances that may buffer against ROS (Gao et al., 2015; Yang et al., 2018). Additionally, as a strong ROS, environmental factors can greatly influence the effectiveness of H_2O_2. For instance, high ultraviolet (UV) intensity and iron will promote the production of ROS, increasing the algaecidal properties of H_2O_2 (Zepp et al., 1992; Drábková et al., 2007). Further testing is needed to better understand the effectiveness of H_2O_2 under a range of environmental conditions commonly observed in industries known to be negatively impacted by poor water quality. This study seeks to fill gaps within the literature by testing the efficacy of H_2O_2 to reduce cyanobacteria under different dissolved organic matter concentrations (humic acid; may impact light availability known to promote ROS production from H_2O_2 or be a carbon source), temperature (which may possibly affect the reaction rate of H_2O_2), and initial levels of algal biomass (possibly affect the amount of H_2O_2 needed for a ‘successful’ treatment). **Methods**

Cultures

Unicellular *Microcystis aeruginosa* (UTEX 2667) used for laboratory experimentation was obtained from the University of Texas at Austin culture collection. Laboratory-cultured *Ankistrodesmus falcatus* was also used for experimentation. Each phytoplankton species was axenically cultured in BG-11 media (Rippka et al., 1979) prior to its use. At the start of each experiment, the cultures were centrifuged to remove overlying BG-11 medium prior to re-suspending cells in filtered (<1.2 µm glass fiber filter) and autoclaved pond water (obtained from farm pond 11 of Auburn University; Boyd and Shelton, 1984).

Dissolved Organic Matter

Four, acid-washed buckets were each filled with 6 L of filtered and autoclaved pond water prior to inoculating each bucket with *M. aeruginosa* (7×10^5 cells mL^{-1}) and *A. falcatus* (1.5×10^4 cells mL^{-1}) to achieve a cell density of *M. aeruginosa* at least one order of magnitude greater than *A. falcatus*. Dipotassium phosphate (K_2HPO_4) and potassium nitrate (KNO_3) were added to each bucket such that total phosphorus (TP) and total nitrogen (TN) concentrations of the autoclaved pond water were 20% greater to that of the ambient water to stimulate phytoplankton growth (Gross and Boyd, 1998). Next, 100% soluble humic acid powder (Earthworks Health®) was added to each bucket to obtain a dissolved organic matter (DOM) concentration of 10, 20, or 60 mg L^{-1} (indicative of the DOM range commonly observed in US freshwater systems; Boyd, 2015). A control with no humic acid added was also used. Each bucket was mixed well to ensure homogenization of phytoplankton and humic acid.

Next, ten, acid-washed, 500 mL glass jars were filled with 435 mL of each DOM treatment concentration. Jars were capped and inverted three times before collecting A/E filtered samples for measurements of algal biomass (as chlorophyll-a, hereinafter called ‘chlorophyll’) and cyanobacterial (as phycocyanin) biomass. Chlorophyll and phycocyanin were measured

fluorometrically (Turner Designs Trilogy®). Chlorophyll was determined by extracting filters in 90% ethanol for 24 hours at 4 ºC (Sartory and Grobbelaar, 1984). Phycocyanin was measured by grinding filters with a pestle and extracting in 50 mM phosphate buffer at room temperature for four hours (Kasinak et al., 2015). After initial algal pigment samples, 395 mL of pond water remained in each jar.

Jars (n=2 per treatment) were then dosed with H_2O_2 (30% concentration by volume; VWR®) at concentrations of 0, 2, 4, 8, or 16 mg L^{-1} (Similar to Yang et al. 2018). To maintain uniform volumes in each jar, secondary stock solutions were made up for each H_2O_2 concentration such that 5 ml of a secondary stock was needed to achieve the overall H_2O_2 concentration in a jar (total jar volume now 400 mL). Upon the addition of the H_2O_2 mixture, jars were inverted 3 times, the caps loosened, and placed in an incubator (Percival® model I-36VL) at 25°C on a 8-hr light:16-hr dark cycle (florescent lighting; intensity = 80 μmol/m^2/s).

Hydrogen peroxide concentrations were measured in each jar daily using the photometric method described by Drábková et al. (2007) until values reached 0 mg L^{-1}. Samples were measured against a blank, which included a whole sample without the addition of horseradish peroxidase. Samples were measured according to the equation:

$$H_2O_2 \text{ sample} = \frac{\Delta A^{551} * V_{final}}{\in L V_{sample}}$$

where ΔA^{551} is the blank-corrected absorbance at 551 nm, ε is the molar extinction coefficient (2100 M^{-1} cm^{-1}), l is the optical cell length in cm (1 cm for this study), V_{sample} is the volume of sample used (0.5 - 7 ml for this study), and V_{final} is the final volume (30.1 ml).

The experiment lasted for seven days. Jars were mixed and rotated within the incubator daily. Chlorophyll and phycocyanin measurements were taken on days 0, 1, 3, 5, and 7.

Temperature

Temperature experiment methods closely followed those described above for the DOM experiment. To start, an acid washed bucket was filled with 7 L of filtered, autoclaved pond water that was enriched with KNO_3 and K_2HPO_4 to reach a concentration of TP and TN 20% greater than ambient water. *M. aeruginosa* (1×10^6 cells mL^{-1}) and *A. falcatus* (1×10^5 cells mL^{-1}) were added to the bucket to achieve a cellular density of *M. aeruginosa* at least one order of magnitude greater than *A. falcatus*. The mixture was stirred well before 435 mL was distributed into each of the 10 glass jars used for each temperature. Jars were then put into an incubator set at either 20, 25, or 30 °C on an 8-hr light:16-hr dark cycle for 24 hours before the start of experiment to acclimate.

To represent day 0, both chlorophyll and phycocyanin samples were taken (day 0 samples) as described above. Jars (n = 2 per treatment) were then dosed with H_2O_2 treatments of 0, 2, 4, 8, or 16 mg L^{-1}, after 24 hrs. To maintain uniform volumes in each of the jars, secondary stock solutions were created for each H_2O_2 treatment and 5 mL of each secondary stock was added to respective jars to reach a total jar volume of 400 mL. Upon the addition of the H_2O_2 mixture, jars were inverted 3 times, the caps loosened, and placed in their temperature specific incubator for the remainder of the seven-day study. Jars were inverted and re-randomized daily to minimize light exposure variation. The 25 and 30 °C trials were conducted simultaneously in different incubators, while the 20°C trial was conducted 2 days thereafter, as only two incubators were available for experimentation. Hydrogen peroxide degradation was measured daily in each jar until values reached zero. Chlorophyll and phycocyanin values were measured on days 1, 3, 5, and 7.

Initial algal biomass

Pond water samples were collected from six active catfish aquaculture ponds at the E.W. Shell Fisheries Center at Auburn University ($1/10^{th}$ acre surface area, 1-meter depth, on average; 500 catfish per pond). The pond water was returned to the lab, filtered through a 500 µm mesh sieve to remove debris, and placed into six separate acid washed buckets. Ponds were chosen based on their phycocyanin and chlorophyll ratios such that a gradient of ponds either dominated by cyanobacteria or other phytoplankton (hereinafter termed 'algae') with varying overall bloom densities were represented (Table 1). Total suspended solids (TSS) of each pond sample were measured using glass filters (GF/C) combusted at 550 °C and pre-weighed before collecting seston and allowing the filters to dry for at least 48 hr at 50 °C (Wilson et al., 2018).

Prior to the start of the experiment, each pond was measured for TP and TN concentrations. Based on these values, KNO_3 and K_2HPO_4 were added the buckets of the respective pond water so that TN and TP values were 20% greater than ambient values. Each bucket was then stirred well and 435 ml of the mixture was added to the jars. Chlorophyll and phycocyanin samples were taken immediately (day 0; A/E filtered samples). Hydrogen peroxide treatments were then added to the jars (n = 2 per treatment) such that each pond water received a concentration of 0, 2, 4, 8 and 16 mg L^{-1} from a secondary stock solution as described above. Jars were then placed on a bench-top (~22.8 °C) in front of fluorescent lights on a 12 hr light:dark schedule (80 jars total). Jars were inverted and location randomized daily. Chlorophyll and phycocyanin were sampled on days 1, 3, 5, and 7.

Statistical analysis

For both the DOM and temperature experiments, a repeated-measures analysis of variance (ANOVA) using a restricted maximum log-likelihood (REML) method was used to assess the effects of H_2O_2, temperature or DOM, and their interaction on algal (chlorophyll) or

cyanobacterial (phycocyanin) biomass over time. Analyses were performed using the **nlme** package in R (Pinheiro et al., 2020; R Core Team, 2017).

In the initial algal biomass experiment, data were analyzed using a generalized linear mixed-model with a binomial distribution using the **lme4** package in R (Bates et al., 2015). Treatments were measured on their ability to reduce phycocyanin concentrations to <20 µg L^{-1} in a binary, yes-no manner. The use of the <20 µg phycocyanin L^{-1} threshold was based on EPA guidelines which states areas having <20 µg chlorophyll L^{-1} are indicative of low-to-moderate issues for harmful algal blooms (USEPA, 2016). This threshold is also been utilized in state lake standards, such as in Wisconsin (WDNR, 2019) and Florida (FDEP, 2013). We utilized phycocyanin values in place of chlorophyll. Predictor variables within our analysis included starting chlorophyll value, TSS, DOM, H_2O_2 treatment (0 - 16 mg L^{-1}), and number of days after application (0 - 7). The pond number was treated as the random effect. Pearson correlations were also drawn between select variables using the **cor.test** package in R (Yu et al., 2020).

Lastly, to indicate to resource managers the concentration of H_2O_2 needed to remove cyanobacteria from a system based on initial algal biomass, the amount of H_2O_2 needed per unit of chlorophyll (i.e., XX mg H_2O_2 L^{-1} /µg chlorophyll L^{-1}) was calculated for each experiment. These values were generated using the H_2O_2 dose applied and starting chlorophyll value in the treatments that reduced phycocyanin values to zero (or nearly-to) over the seven day studies.

Results

Dissolved Organic Matter

Hydrogen peroxide was fully degraded in all four DOM concentrations within 48-hr at 2 and 4 mg H_2O_2 L^{-1}, 72-hr at 8 mg H_2O_2 L^{-1} (measured to 120-hr, but values were negligible), and 168-hr at 16 mg H_2O_2 L^{-1} (Figure 4.1). Although variation across DOM concentrations was

small, statistically significant differences in H_2O_2 degradation rates among DOM concentrations were observed at 4 ($F = 10.7$, $p = 0.02$) and 16 mg H_2O_2 L^{-1} ($F = 25.8$, $p = 0.004$). At these treatments, H_2O_2 degraded the slowest at lower DOM concentrations. At 16 mg H_2O_2 L^{-1}, a slight increase in H_2O_2 was observed between 48 - 72-hr in the jars without added DOM. Significant differences in H_2O_2 between DOM concentrations were not observed at 2 ($F = 2.6$, $p = 0.19$) or 8 mg H_2O_2 L^{-1} ($F = 3.1$, $p = 0.15$).

Marginally significant differences in algal biomass (as chlorophyll) were observed among DOM concentrations at 0 ($F = 5.5$, $p = 0.067$), 2 ($F = 5.8$, $p = 0.062$), and 4 mg H_2O_2 L^{-1} ($F = 6.03$, $p = 0.058$) (Figure 4.2). Algal biomass increased over the 7-day trial at 0, 2, and 4 mg H_2O_2 L^{-1} (39, 47, and 48%, respectively, across all DOM concentrations). Algal biomass decreased in all DOM concentrations at 8 (85%) and 16 (88%) mg H_2O_2 L^{-1}, and significant differences among DOM concentrations were observed [($F = 7.4$, $p = 0.042$), ($F = 56.9$, $p = 0.001$), respectively]. In the 8 and 16 mg H_2O_2 L^{-1} treatments, the greatest decreases algal biomass were observed in jars receiving lower DOM concentrations, with the 60 mg DOM L^{-1} concentration having a significantly greater algal biomass than 0 mg DOM L^{-1} jars after 7 days ($p \leq 0.05$).

A significant difference between cyanobacterial biomass (as phycocyanin) with respect to DOM concentration was observed at 0 ($F = 15.4$, $p = 0.011$) and 2 mg H_2O_2 L^{-1} ($F = 17.1$, $p = 0.0096$) (Figure 4.3). At these H_2O_2 treatments, it was observed that cyanobacterial density increased over the seven days with greater DOM concentration (173 and 249% increase across all DOM concentrations, respectively), especially in the control jars receiving no H_2O_2. This relationship to DOM concentration were opposite to the trends for chlorophyll at 0 H_2O_2 mg L^{-1} (Figure 4.2). Cyanobacteria decreased over the 7-day trial at 4 ($F = 2.5$, $p = 0.20$; although values did increase slightly by day seven), 8 ($F = 0.4$, $p = 0.75$), and 16 mg H_2O_2 L^{-1} ($F = 0.9$, $p = 0.52$),

with no observed significant differences among DOM concentrations. This indicated that a treatment range of 0.03 - 0.12 mg H_2O_2 L^{-1} /µg chlorophyll L^{-1} was effective at reducing cyanobacterial densities to, or nearly-to, zero regardless of DOM concentration.

Temperature

Hydrogen peroxide fully decomposed in all three temperature trials by 24-hr at 2 and 4 mg L^{-1}, 48-hr in 8 mg L^{-1}, and 120-hr in 16 mg L^{-1} (H_2O_2 present at 20 ºC, but minimally; Figure 4.4). Significant differences among H_2O_2 degradation and temperature were observed in all H_2O_2 treatments tested [2 ($F = 11.2$, $p = 0.0094$), 4 ($F = 115.0$, $p < 0.0001$), 8 ($F = 75.3$, $p = 0.0001$), and 16 mg H_2O_2 L^{-1} ($F = 40.2$, $p = 0.0003$)]. Despite being completely removed at similar time points across temperatures, it was typically observed that H_2O_2 degraded more slowly at 20 °C, the lowest temperature tested, in each H_2O_2 treatment.

A decrease in algal biomass (as chlorophyll) was observed in all H_2O_2 treatments and the degree of reduction was inversely related to H_2O_2 concentration (32.3, 41.3, 45.6, 86.1, 98.4% decrease in the 0, 2, 4, 8, and 16 mg H_2O_2 L^{-1}, respectively; Figure 4.5). Significant differences among algal biomass and temperature were observed at 0 ($F = 9.2$, $p = 0.015$), 8 ($F = 40.0$, $p = 0.0003$), and 16 mg H_2O_2 L^{-1} ($F = 364.8$, $p < 0.0001$). In these H_2O_2 treatments, the jars at 20 °C had the greatest algal biomass remaining after seven days. Lastly, 2 ($F = 4.4$, $p = 0.066$) and 4 mg H_2O_2 L^{-1} ($F = 0.5$, $p = 0.65$) indicated no significant difference in algal biomass among temperatures.

The effectiveness of H_2O_2 on cyanobacteria (as phycocyanin) varied among temperatures and treatments (Figure 4.6). Significant differences in cyanobacterial biomass and temperature were observed at 0 ($F = 24.6$, $p < 0.0001$), 2 ($F = 4.3$, $p = 0.025$; although Tukey's post-hoc test observed marginal significance among treatments), and 8 mg H_2O_2 L^{-1} ($F = 7.6$, $p=0.003$). The 4

(F = 1.3, p = 0.30) and 16 mg H_2O_2 L^{-1} (F = 1.7, p = 0.21) treatments were found to have no significant difference in effectiveness between temperatures. Jars receiving 0 mg L^{-1} H_2O_2 had a relatively constant amount of cyanobacteria over the course of the experiment, with the exception of 32 °C treatments that dropped in density significantly on day 5 (85% decrease from day 3 to 5). Hydrogen peroxide reduced cyanobacterial densities to nearly zero over the 7-day experiment with treatments of 8 and 16 mg H_2O_2 L^{-1}, equating a treatment of 0.04 - 0.09 mg H_2O_2 L^{-1} /µg L^{-1} chlorophyll at temperatures between 20 and 32 °C.

Initial algal biomass

Water from the six catfish aquaculture ponds used for this trial contained varying starting densities of cyanobacteria and other algae; however, given that these systems were largely dominated by cyanobacteria, a strong relationship between chlorophyll (measure of algal biomass) and phycocyanin (measure of cyanobacterial biomass) values were observed (Pearson's r = 0.84, p = 0.04; Table 4.1). Overall, chlorophyll and phycocyanin values ranged from 82.0 – 371.8 µg L^{-1} and 78.2 – 1060.7 µg L^{-1}, respectively. TSS was positively correlated with chlorophyll and phycocyanin (r = 0.79, p = 0.06 and r = 0.49, p = 34, respectively). DOM was negatively correlated with chlorophyll (r = -0.36, p = 0.49); however, there was no relation between DOM and phycocyanin (r =-0.03, p = 0.95).

A generalized linear mixed model with a binomial distribution was used to describe conditions needed to reduce cyanobacterial biomass (≤20 µg L^{-1} phycocyanin). Of the variables measured, H_2O_2 treatment concentration (z = 4.9, p <0.0001), starting chlorophyll biomass (z = -3.6, p <0.0001) and days after treatment (z = 3.9, p <0.0001) were the best predictors of a treatment reducing cyanobacteria to ≤20 µg L^{-1}. The resulting equation is as follows:

$$Phy(<20ug/L) = \frac{exp^{(-0.95+0.24(H2O2)+0.42(Days)-0.015(Chlorophyll\ Starting\ Density))}}{1 + exp^{(-0.95+0.24(H2O2)+0.42(Days)-0.015(Chlorophyll\ Starting\ Density))}}$$

where H_2O_2 = 0 - 16 mg L^{-1}, days = 0 - 7, and chlorophyll starting concentration = 122 - 371 µg L^{-1}. The model indicates that the higher the initial algal biomass (as chlorophyll), the less likely it is that a treatment would be able to reduce cyanobacteria to ≤20 µg phycocyanin L^{-1}. But, higher the H_2O_2 treatment concentration, and the more time has passed since a treatment, the probability that the treatment will reduce phycocyanin values to ≤20 µg L^{-1} increases.

Discussion

Although a tremendous amount of research has been devoted to assessing how environmental conditions may contribute to cyanobacterial blooms in freshwater systems (Paerl et al. 2001), there has been little focus on how these factors may affect mitigation strategies such as chemical treatments. Physical and chemical water quality conditions might also affect the efficiency of treatments and if/when/how a cyanobacterial bloom will reoccur after a treatment (Drábková et al. 2007). In total, this study contributes to our understanding of the physical and chemical influences on the efficacy of hydrogen peroxide by evaluating the influence of DOM, water temperature, and initial algal biomass at the time of treatment.

Following the methods of past studies, H_2O_2 was tested in this study under laboratory conditions, allowing for increased replication and ideal study conditions (Drábková et al., 2007; Yazizi and Deveci 2010; Greenfield et al. 2014; Crafton et al. 2019). Environmental conditions, application methods, mixing, and overall project scaling may influence treatment outcomes when H_2O_2 is applied in the field. We hope the outcomes of this study provide resource managers additional factors to consider when determining the success of a H_2O_2 treatment. The findings of the DOM, temperature, and initial algal biomass experiments will subsequently be described in detail.

Dissolved Organic Matter (humic acid)

Dissolved organic matter in freshwater systems is generated from the breakdown of organic material from allochthonous and autochthonous sources, which numerous and diverse factions of compounds existing (Boyd, 2015). A study of dissolved organic carbon in North American freshwater systems indicates that average concentrations increased by about 0.15 mg L^{-1} between 1990 to 2004; rising CO_2 levels, increased runoff, and climate change all likely contributed to this increase (Reitsema et al., 2018). Dissolved organic matter can influence primary productivity in these systems in both a beneficial and deleterious manner. For instance, DOM can reduce productivity by decreasing light attenuation, but can increase productivity by providing a nutritional baseline for bacteria, including cyanobacteria (Reitsema et al., 2018). In this way, DOM is an example a water quality variable that may affect the outcomes of an algaecide treatment (e.g., reduced light and increased organic matter), and is also of a variable that may change following a treatment (e.g., increased dissolved nutrients and less algal biomass).

In this series of laboratory experiments, DOM had no significant effect on the ability of ≥4 mg H_2O_2 L^{-1} to reduce total cyanobacterial biomass over the course of seven days (a H_2O_2 dosing rate of 0.03 - 0.12 mg H_2O_2 L^{-1} /µg L^{-1} chlorophyll). This finding contrasts with our hypothesis that H_2O_2 would react to the humic substances in the water (Doskočil et al., 2014), reducing H_2O_2 concentrations more rapidly than in low DOM waters. Increased light attenuation caused by elevated DOM concentrations was also expected to reduce the effectiveness of H_2O_2, since UV light exposure may compound the effects of H_2O_2 on cyanobacteria (Drábková et al., 2007). Algal biomass (measured as chlorophyll) were significantly higher in the treatments with 60 mg DOM L^{-1} when given treatment of 8 and 16 mg H_2O_2 L^{-1}, however, suggesting that low UV light exposure and/or elevated humic substance content may have reduced the effectiveness

of H_2O_2. The DOM concentrations used in this study represented a broad range of conditions from clear (≤10 mg L^{-1}) to those indicative of swamps or polluted systems (≥50 mg L^{-1}; Boyd, 2015). As this environmentally relevant range of DOM values did not reduce the efficacy of H_2O_2, it reflects the potential usefulness of H_2O_2 in highly colored systems, such as wastewater (Barrington et al. 2008) or aquacultural industries (Sinha et al., 2018).

At 16 mg L^{-1}, H_2O_2 persisted longer in the treatment without DOM. It is probable that additional H_2O_2 persisted in the 16 mg L^{-1} treatments and was further generated by the increased light penetration in the low DOM conditions. Further, as no algae remained at 8 mg H_2O_2 L^{-1}, it is possible that there was H_2O_2 still present in the 16 mg H_2O_2 L^{-1} treatment that persisted for an extended period of time. Interestingly, it was found that H_2O_2 persisted longer in this series of experiments than it did in the temperature experiments. This could be due the lower cell concentrations used in the DOM experiments (see methods for details).

A substantial increase in cyanobacterial biomass (as phycocyanin) was observed at 60 mg L^{-1} DOM with H_2O_2 treatments ≤2 mg L^{-1}. The increase of cyanobacteria in the presence of DOM has been documented previously (Paerl et al., 2001). Dissolved organic matter uptake may directly occur by cyanobacteria for heterotrophic growth, or support the non-photosynthetic bacterial community within a cyanobacterial bloom; a process that occurs in both freshwater and marine systems (Paerl et al., 1993; Znachor and Nedoma, 2010). The uptake of dissolved organic carbon by phytoplankton can be extremely important in low light systems or at night when photosynthesis does not occur (Znachor and Nedoma, 2010). DOM may also increase in systems experiencing large blooms as a result of cellular decay processes (Paerl et al., 2001), causing debate as to whether DOM is a cause or a consequence of cyanobacterial blooms. Although it is

likely that DOM is both a product of, and contributor to, cyanobacterial blooms, our findings lend support to the hypothesis that DOM promotes cyanobacterial growth.

Temperature

In North America, cyanobacterial blooms are typically observed in the late spring-early fall, coinciding with long days and warmer temperatures (Paerl and Huisman, 2008). The temperature range assessed in these experiments represents ambient North American water temperatures during late spring-early fall (20 - 32 °C; mean temperature of 1147 USA lakes sampled in the summer months = 24 °C; Beaulieu et al., 2013). Temperatures ≥25 °C will often promote cyanobacterial growth, although numerous other factors also affect bloom formation (Paerl and Huisman, 2008). Our results indicate that H_2O_2 decomposed significantly faster at 32 °C when H_2O_2 was ≥4 mg L^{-1}; however, no significant differences in the reduction of cyanobacteria were observed across water temperatures at these H_2O_2 treatments. Yazici and Deveci, (2010) also observed small but significant differences in H_2O_2 degradation between 20 and 30 °C over a three-hour period in a study using H_2O_2 treatments of 800 mg L^{-1}. Although higher temperatures do increase the decomposition rate of H_2O_2 (Yazici and Deveci, 2010), it is likely that H_2O_2 reacts with organic and inorganic matter, including cyanobacteria, in the water column occur rapidly (Barrington et al., 2008), negating the importance of prolonged H_2O_2 degradation over many hours to days.

Hydrogen peroxide was not observed to have a different effect at reducing algal or cyanobacterial biomass across the three temperatures tested. A significant difference in the reduction of algal biomass was observed between 20 and 25 °C, but values were reduced to nearly zero at both temperatures by the end of 7 days. Overall, H_2O_2 treatments of 8 or 16 mg L^{-1} were effective at reducing cyanobacteria and phytoplankton to nearly 0 over the seven-day trial

(a H_2O_2 dosing rate of 0.04-0.09 H_2O_2 mg L^{-1}/µg L^{-1} chlorophyll). It is possible that the decomposition times of H_2O_2 at higher water temperatures (>32 °C) may negatively affect its value as an algaecide and should be researched further. In general, the temperature range used in this study would reflect the effectiveness of H_2O_2 in a typical North American lake or reservoir during late spring-early fall.

Initial Algal Concentration

The ability of H_2O_2 to reduce cyanobacteria was tested on water from several ponds containing a wide range of cyanobacteria to phytoplankton ratios and initial algal concentrations. To reduce phycocyanin to ≤20 µg L^{-1}, it was found that initial H_2O_2 concentration, number of days after application, and algal biomass (as indicated by chlorophyll) were significant predictors to determine the probability of success. Both H_2O_2 concentration and days after application had a positive effect on treatment success, while initial algal biomass had a negative effect. The time it takes for cyanobacteria to be reduced by 50% after a H_2O_2 treatment often takes only hours, and cyanobacteria may reach negligible values in days (Barrington et al., 2008). Likewise, this study found that the cyanobacteria (as indicated by phycocyanin) decreased over the seven-day trial, with the greatest reduction occurring typically within 24 hours at ≥4 mg H_2O_2 L^{-1}. Such concentrations have also been observed to reduce cyanobacterial densities within 24 hours in prior studies (Lurling et al., 2014; Yang et al., 2018).

Of the three variables found to be significant in predicting the probability of reducing cyanobacteria, initial algal biomass had the strongest and negative influence on treatment success. This finding was expected as more phytoplankton will require a larger portion of H_2O_2 to neutralize the increased biomass (Barrington et al., 2008). However, recommendations for treating a cyanobacteria based on ambient algal biomass are rarely provided in the literature,

although Barrington et al. (2011) recommended found that a treatment of 0.11 mg H_2O_2 L^{-1}/µg chlorophyll L^{-1} was effective. In the treatments having the densest algal biomass (371.8 µg L^{-1} chlorophyll), 0.04 mg H_2O_2 L^{-1}/µg chlorophyll L^{-1} was effective at reducing phycocyanin values to ≤20 µg L^{-1} after seven days, although phycocyanin values did increase to 111.64 µg L^{-1} by day seven. Based on the three experiments encompassed by this study (i.e., temperature, DOM, initial algal concentration), treatments of 0.03 to 0.12 mg H_2O_2 L^{-1}/µg chlorophyll L^{-1} would be effective.

Although chlorophyll is relativity easy to measure and is widely used as an indicator of overall algal biomass, it should be noted that chlorophyll is a pigment that can be found in many species of phytoplankton. As such, a delineation cannot be made between cyanobacteria and other phytoplankton based on chlorophyll measurements alone. Phycocyanin, a pigment found largely in cyanobacteria, may also be measured to help estimate the abundance of cyanobacteria (Kasinak et al., 2015). While it has been found that the biomass of cyanobacteria will affect treatment outcomes (as has been found to be true for other control types such as biomanipulation; Sarnelle, 2007), it is also possible that other phytoplankton species may hinder the effectiveness of H_2O_2. For example, it was found in this study and other publications (Yang et al., 2018) that H_2O_2 have a reduced affect to chlorophytes as it does cyanobacteria. Moreover, differences in susceptibility will occur among different cyanobacterial genera (Yang et al., 2018), and treatment effectiveness may be context specific based ambient taxa. Further testing is needed to delineate how bloom dominance will affect H_2O_2 treatments.

Conclusions

The use of H_2O_2 to combat cyanobacteria is a possible alternative to other algaecides more routinely used. Understanding how biotic and abiotic factors affect the ability of H_2O_2 to

control phytoplankton is required. This study assessed how DOM, temperature, and initial algal biomass impacted the effectiveness of H_2O_2 as an algaecide, specifically to cyanobacteria. Although DOM concentration and water temperature affected the degradation rate of H_2O_2, both had little effect on the ability of H_2O_2 to reduce cyanobacterial densities. These findings indicate that H_2O_2 will be effective in conditions typically observed in North American lakes and reservoirs. In this series of experiments, it was observed that that 0.03 - 0.12 mg H_2O_2 L^{-1} /µg chlorophyll L^{-1} (i.e., the 4, 8, or 16 mg L^{-1} H_2O_2 treatments) were effective a fully removing cyanobacteria.

Initial algal biomass (based on chlorophyll values), H_2O_2 concentration, and days after treatment were all found to be significant predictors in determining the probability of H_2O_2 treatments reducing cyanobacteria to low densities (i.e., ≤20 µg L^{-1} phycocyanin) across a wide range of ambient algal biomasses. Of these variables, initial algal biomass had the greatest negative influence on the effectiveness of H_2O_2. In the densest blooms tested in this study, it was observed that 0.04 mg H_2O_2 L^{-1}/µg chlorophyll L^{-1} was needed to reduce phycocyanin to ≤20 µg L^{-1}. It should be noted that while chlorophyll is an easily measured variable and an excellent predictor of phytoplankton, its value alone does not necessarily indicate the abundance of cyanobacteria. Combining chlorophyll measures with other pigments or cell counts to determine the presence of cyanobacteria in an algal bloom is needed first to determine if an algaecide is needed and how effective it may be.

Acknowledgements

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Tables and Figure Legends

Table 4.1. Water quality measurements, including starting phycocyanin to chlorophyll ratio, chlorophyll, phycocyanin, total suspended solids (TSS), and dissolved organic matter (DOM), of the six ponds used in the experiment focused testing the influence of initial algal bloom concentrations on the effectiveness of H_2O_2.

Figure 4.1. Degradation of (A) 2, (B) 4, (C) 8, and (D) 16 mg L^{-1} hydrogen peroxide (H_2O_2) over a seven-day lab study at dissolved organic matter (DOM) concentrations of 0, 10, 20, and 60 mg L^{-1} (n = 2). Error bars represent one standard error. Letters after each treatment are results

from Tukey's multiple comparison tests. Treatments sharing the same letter are not statistically different ($p \leq 0.05$).

Figure 4.2. Dynamics of algal biomass (measured as chlorophyll-*a*) when treated with (A) 0, (B) 2, (C) 4, (D) 8, or (E)16 mg H_2O_2 L^{-1} over a seven-day lab study at dissolved organic matter (DOM) concentrations of 0, 10, 20, and 60 mg L^{-1} (n=2). Error bars represent one standard error. Letters after each treatment are results from Tukey's multiple comparison tests. Treatments sharing the same letter are not statistically different ($p \leq 0.05$).

Figure 4.3. Dynamics of cyanobacteria (measured as phycocyanin) when treated with (A) 0, (B) 2, (C) 4, (D) 8, or (E)16 mg H_2O_2 L^{-1} over a seven-day lab study at dissolved organic matter (DOM) concentrations of 0, 10, 20, and 60 mg L^{-1} (n = 2). Error bars represent one standard error. Letters after each treatment are results from Tukey's multiple comparison tests. Treatments sharing the same letter are not statistically different ($p \leq 0.05$).

Figure 4.4. Degradation of (A) 2, (B) 4, (C) 8, and (D) 16 mg H_2O_2 L^{-1} over a seven-day lab study at 20, 25, and 32 ºC (n = 3). Letters after each treatment are results from Tukey's multiple comparison tests. Error bars represent one standard error. Treatments sharing the same letter are not statistically different ($p \leq 0.05$).

Figure 4.5. Dynamics of algal biomass (measured as chlorophyll) when treated with (A) 0, (B) 2, (C) 4, (D) 8, or (E) 16 mg H_2O_2 L^{-1} at 20, 25, or 32 ºC (n = 3) over a seven-day lab study. Error bars represent one standard error. Letters after each treatment are results from Tukey's multiple comparison tests. Treatments sharing the same letter are not statistically different ($p \leq 0.05$).

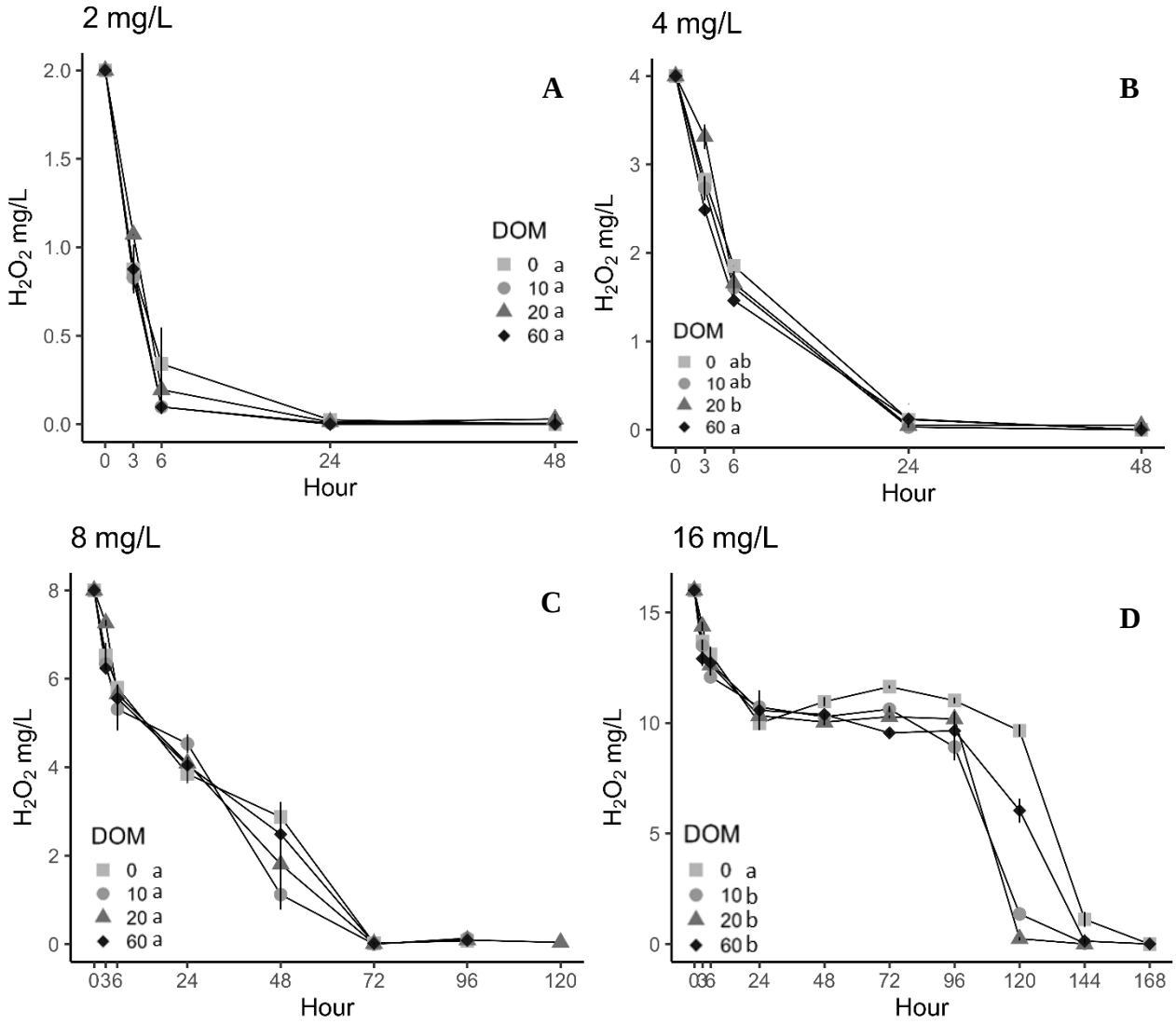
Figure 4.6. Dynamics of cyanobacteria (measured as phycocyanin) when treated with (A) 0, (B) 2, (C) 4, (D) 8, or (E) 16 mg H_2O_2 L^{-1} at 20, 25, or 32 ºC (n = 3) over a seven-day lab study.

Error bars represent one standard error. Letters after each treatment are results from Tukey's multiple comparison tests. Treatments sharing the same letter are not statistically different ($p \leq 0.05$).

Table 4.1

Phycocyanin: chlorophyll ratio	Chlorophyll µg L^{-1}	Phycocyanin µg L^{-1}	TSS mg L^{-1}	DOM mg L^{-1}
0.8	162.5	124.6	74.5	45.8
0.8	122.4	98.1	101	57
1.0	82.03	78.2	48.8	60
1.2	253.3	294.6	205.1	44.7
2.3	233.4	546.4	129.1	47.7
2.9	371.8	1060.7	162.3	54.5

Figure 4.1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A
H_2O_2 mg/L
Hour
DOM
0 a
10 a
20 a
60 a
4 mg/L
B
H_2O_2 mg/L
Hour
DOM
0 ab
10 ab
20 b
60 a
8 mg/L
C
H_2O_2 mg/L
Hour
DOM
0 a
10 a
20 a
60 a
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D
H_2O_2 mg/L
Hour
DOM
0 a
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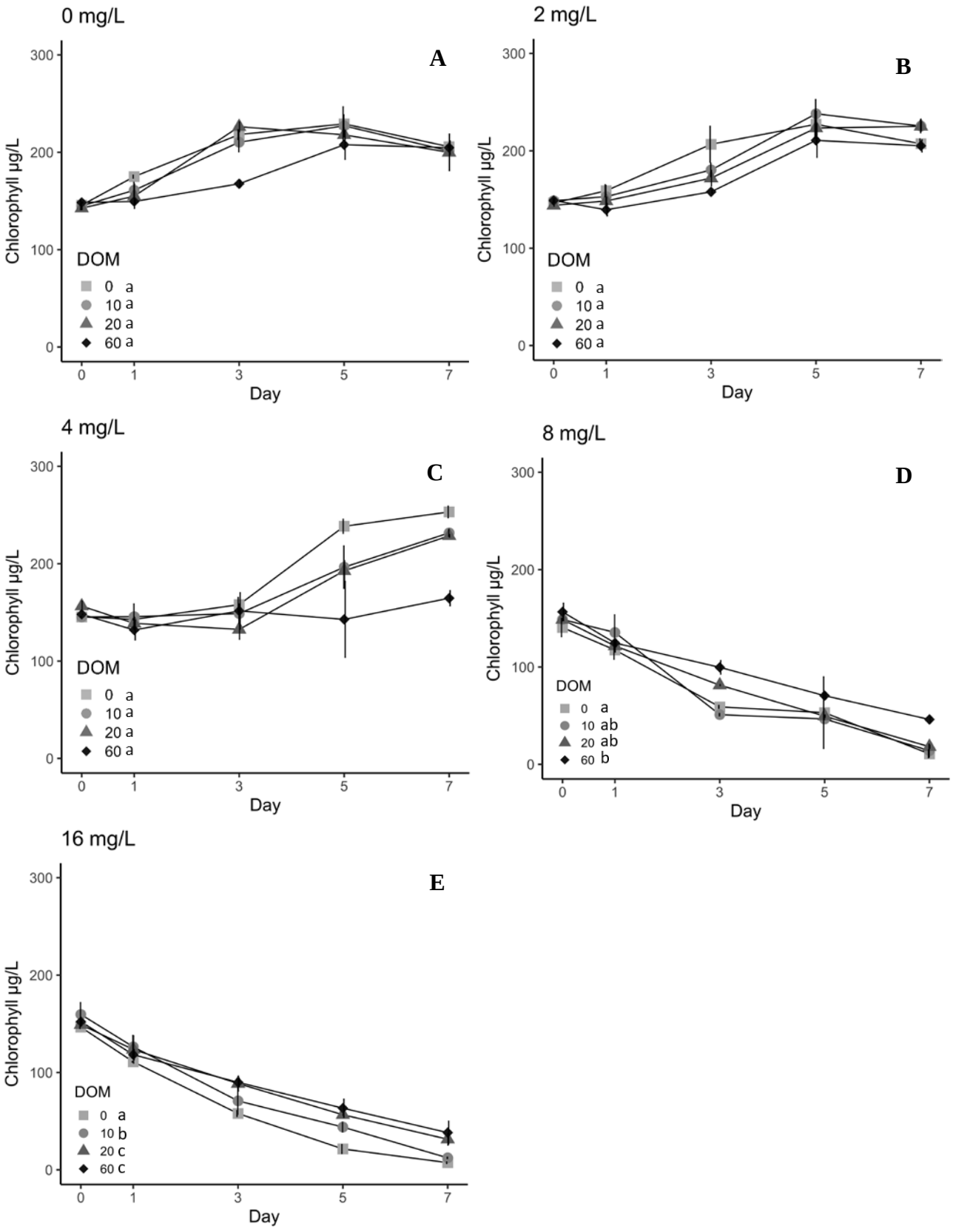
20 b
60 b

Figure 4.2


0 mg/L
A
2 mg/L
B
4 mg/L
C
8 mg/L
D
16 mg/L
E
Chlorophyll µg/L
Day
DOM
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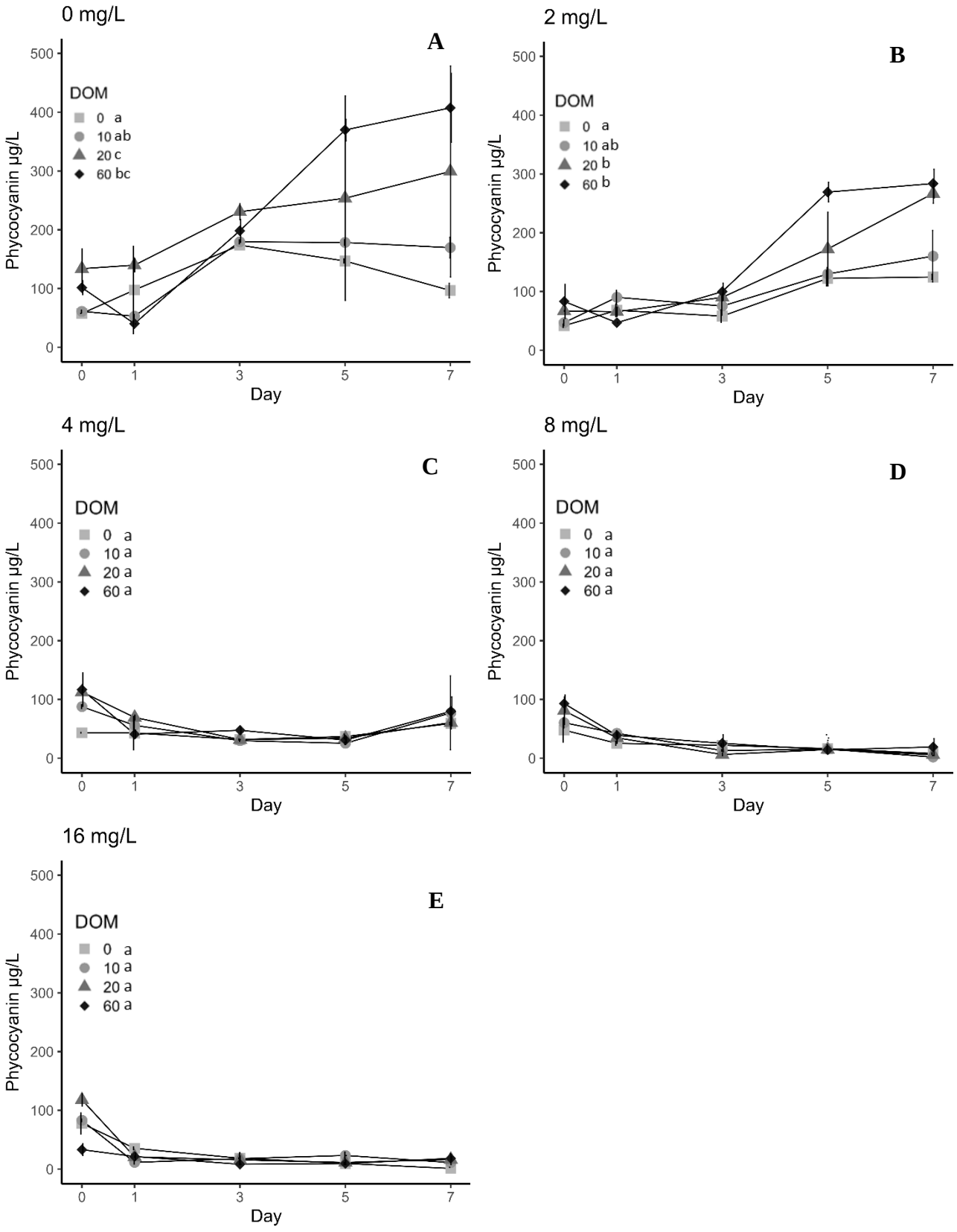
20 a
60 a
0 a
10 ab
20 ab
60 b
0 a
10 b
20 c
60 c

Figure 4.3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DOM
0 a
10 ab
20 c
60 bc
2 mg/L
B
DOM
0 a
10 ab
20 b
60 b
4 mg/L
C
DOM
0 a
10 a
20 a
60 a
8 mg/L
D
DOM
0 a
10 a
20 a
60 a
16 mg/L
E
DOM
0 a
10 a
20 a
60 a
Phycocyanin µg/L
Day
0
100
200
300
400
500
0
1
3
5
7

Figure 4.4

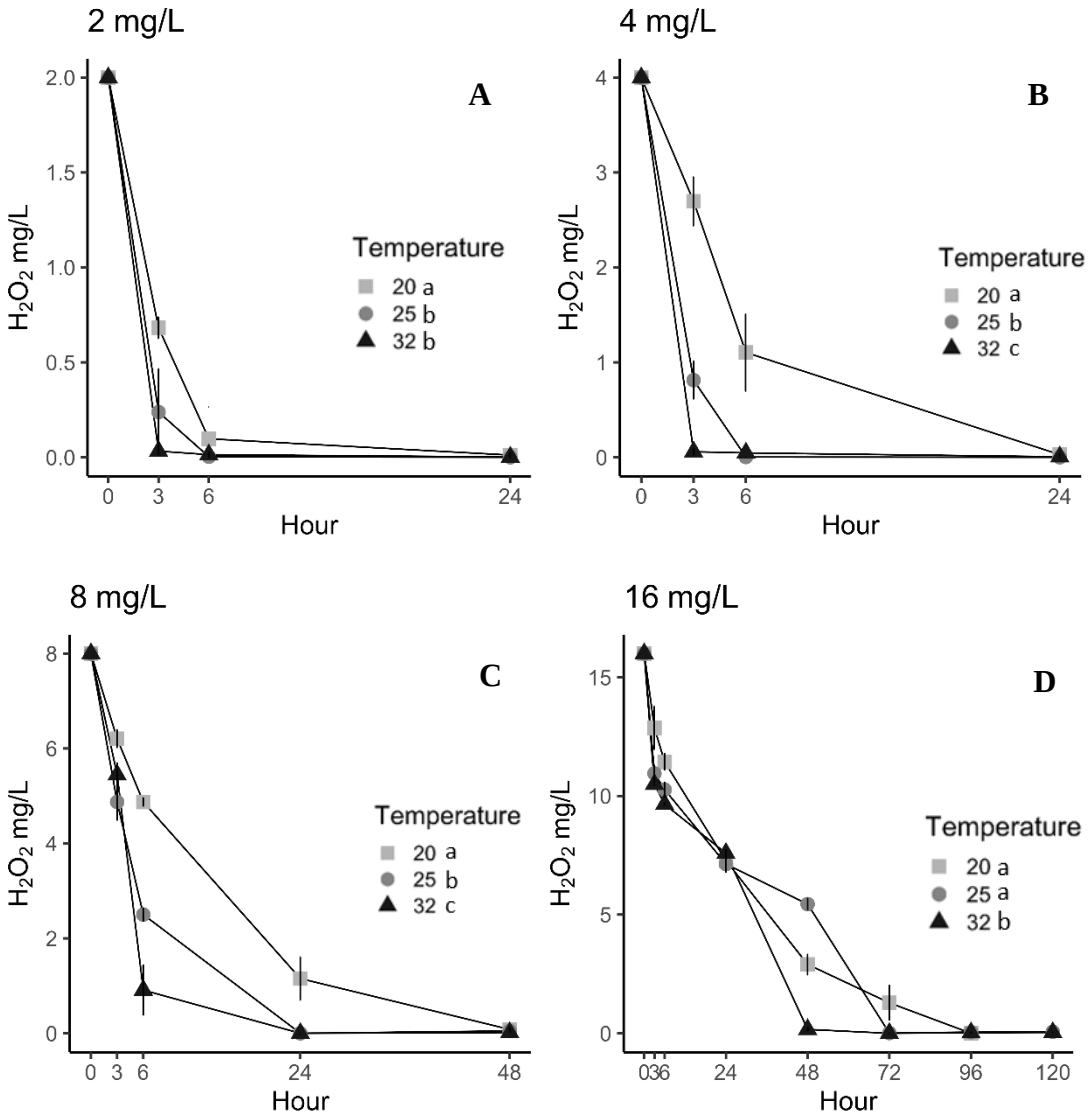

2 mg/L
A
H_2O_2 mg/L
Hour
Temperature
20 a
25 b
32 b
4 mg/L
B
H_2O_2 mg/L
Hour
Temperature
20 a
25 b
32 c
8 mg/L
C
H_2O_2 mg/L
Hour
Temperature
20 a
25 b
32 c
16 mg/L
D
H_2O_2 mg/L
Hour
Temperature
20 a
25 a
32 b

Figure 4.5

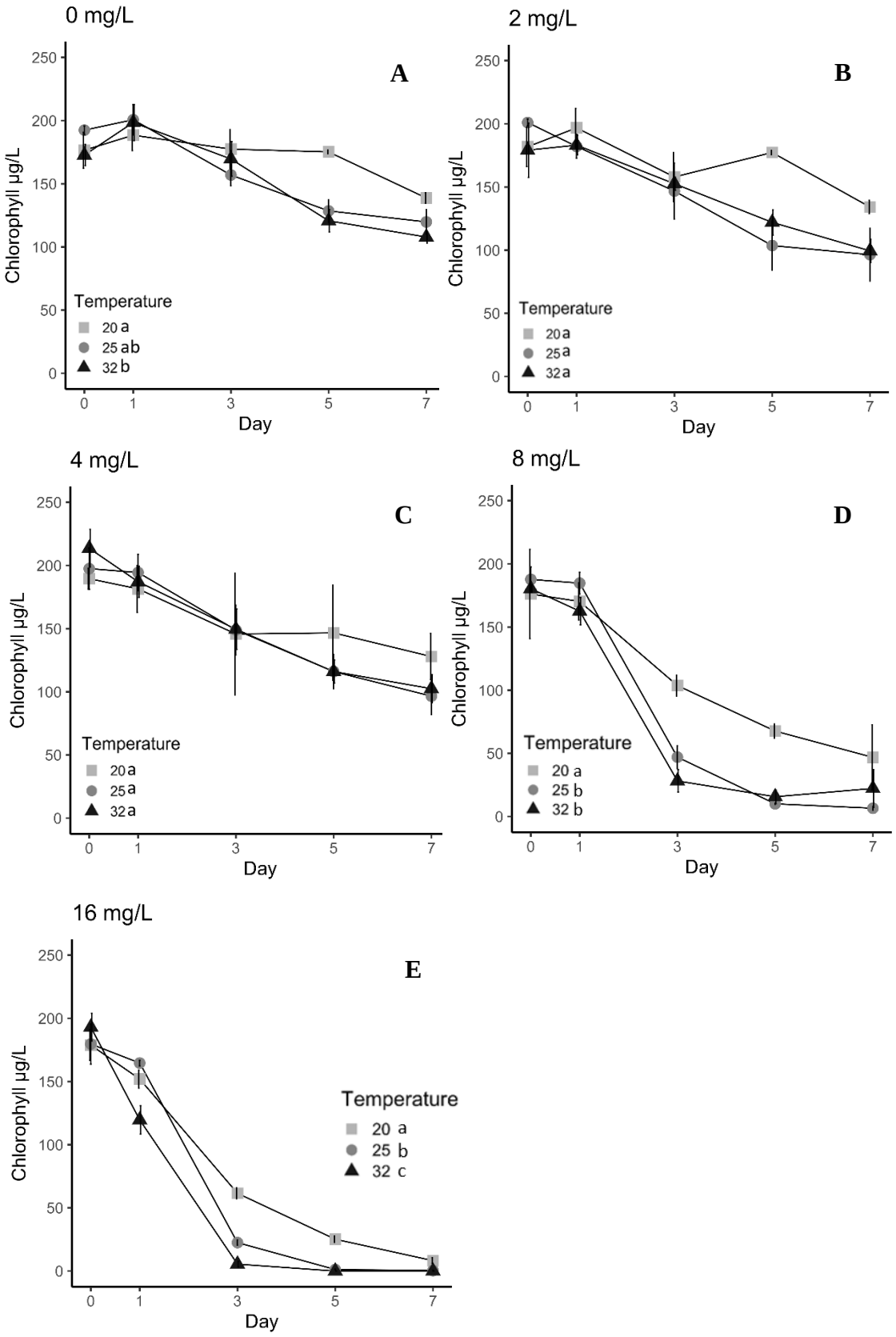
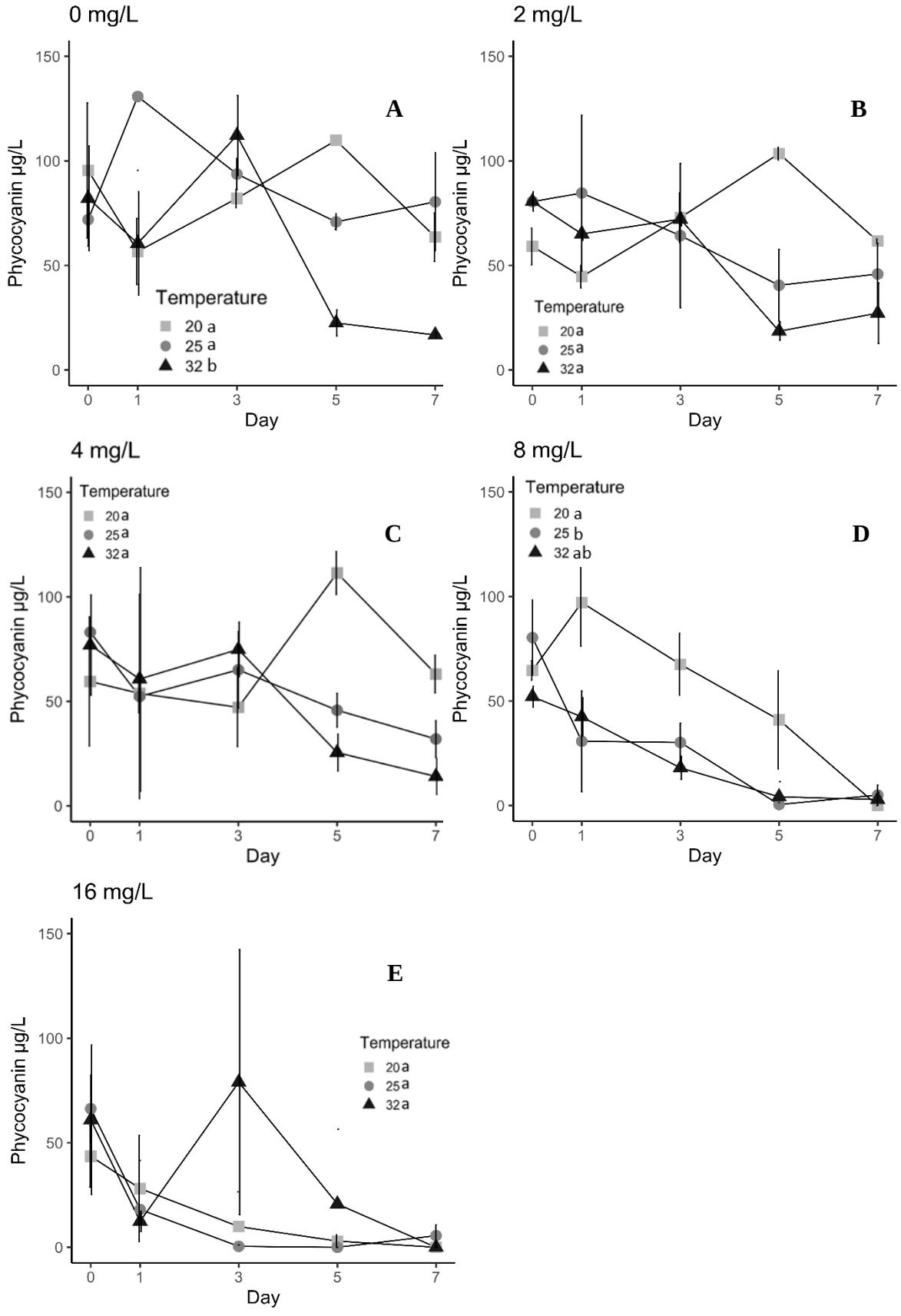

0 mg/L
A
Chlorophyll µg/L
Temperature
20 a
25 ab
32 b
Day
2 mg/L
B
Temperature
20 a
25 a
32 a
Day
4 mg/L
C
Temperature
20 a
25 a
32 a
Day
8 mg/L
D
Temperature
20 a
25 b
32 b
Day
16 mg/L
E
Temperature
20 a
25 b
32 c
Day

Figure 4.6


0 mg/L
A
Phycocyanin µg/L
Day
Temperature
20 a
25 a
32 b
2 mg/L
B
Temperature
20a
25a
32a
4 mg/L
C
Temperature
20a
25a
32a
8 mg/L
D
Temperature
20 a
25 b
32 ab
16 mg/L
E
Temperature
20a
25a
32a

Appendix A. (Chapter 1)

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Supplementary tables and figures

Figure A1

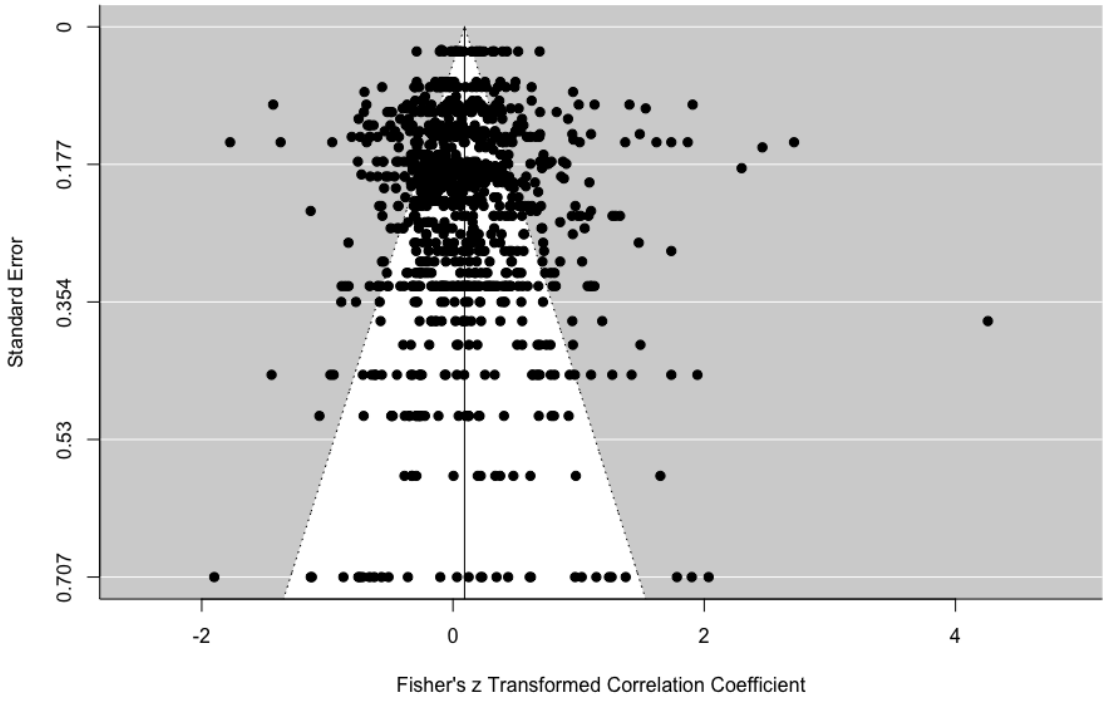

Table A1

Symbol	Meaning	Action
<	Less than	Half-values assigned, as performed by Orihel, et al. (2012)
E	Estimated values	Values kept
M	Presences verified, but not quantified	Values kept
V	Volume affected by contamination	Values removed

Table A2

Parameter	Effect (*r*)	LCI	UCI	Studies	I^2 (%)
Temperature*	0.20	0.11	0.28	106	87
Turbidity	0.18	-0.03	0.38	34	96
Total Suspended Solids	0.08	-0.04	0.18	21	48
Conductivity	0.07	-0.02	0.17	69	85
Secchi Depth*	-0.20	-0.30	-0.09	44	91

Table A3

Parameter	Effect (*r*)	LCI	UCI	Studies	I^2(%)
Chlorophyll*	0.45	0.31	0.57	76	97
Silicon Dioxide	0.14	-0.22	0.47	4	88
pH*	0.13	0.02	0.23	92	89
Hardness	0.11	-0.02	0.25	22	0
Total Organic Carbon	0.08	-0.09	0.25	15	68
Bicarbonate Filtered	0.07	-0.13	0.27	15	1
Dissolved Organic Carbon*	0.07	0.03	0.11	17	0.02
Sulphate	0.02	-0.02	0.06	3	0
Carbon Dioxide	-0.06	-0.19	0.08	24	0
Dissolved Oxygen	-0.09	-0.20	0.03	74	86
Alkalinity	-0.26	-0.53	0.05	14	76

Table A4

Parameter	Effect (*r*)	LCI	UCI	Studies	I^2 (%)
Total Kjeldahl Nitrogen	0.30	-0.40	0.78	5	98
Total Dissolved Phosphorus *	0.30	0.07	0.50	13	89
Total Phosphorus*	0.22	0.13	0.30	92	91
Ammonia as Organic Nitrogen, Unfiltered	0.21	-0.02	0.42	21	75
Ammonia as Organic Nitrogen	0.17	-0.06	0.39	12	35
Phosphate*	0.14	0.00	0.28	59	89
Particulate Nitrogen	0.10	-0.10	0.30	5	0
Ammonium	0.08	-0.09	0.25	30	93
Total Organic Nitrogen	0.07	-0.12	0.25	35	72
Soluble Reactive Phosphorus	0.05	-0.10	0.20	31	65
Nitrite	0.03	-0.08	0.14	47	74
Nitrite and Nitrate	0.03	-0.16	0.21	38	94
Total Dissolved Nitrogen	0.01	-0.18	0.20	13	83
Total Nitrogen	-0.01	-0.10	0.08	82	90
Dissolved Organic Nitrogen	-0.01	-0.21	0.18	24	61
Ammonia	-0.02	-0.30	0.27	6	94
Ammonia + Ammonium	-0.07	-0.17	0.04	34	12
Nitrate*	-0.12	-0.20	-0.04	74	80
Dissolved Nitrogen*	-0.19	-0.35	-0.02	5	0

Appendix B. (Chapter 2)

Supplementary references

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USGS 314458093085101 Sibley Lake west branch about 0.5 miles east of br Natchitoches Parish, Louisiana

USGS 402003081095303 Tappan Lake at Scio OH

USGS 07144790 CHENEY RE NR CHENEY, KS

USGS 02154950 LAKE WILLIAM C. BOWEN NEAR FINGERVILLE SC

USGS 06857050 MILFORD LAKE NR JUNCTION CITY KS

USGS 06892450 OLATHE LK NR OLATHE KS

USGS 07369647 LAKE ST. JOSEPH NR NEWELLTON LA

USGS 07369648 LK BRUIN AT LK BRUIN ST PARK LA

USGS 07381424 FALSE R @ NEW ROADS LA

USGS 08067074 CWA Canal at Thompson Rd nr Baytown TX

USGS 294607095042700 Lynchburg Res nr Intake Structure nr Baytown tx

USGS 294643095035200 Lynchburg Res nr CWA Canal Inflow nr Baytown tx

USGS 295510095084801 Lk Houston Site C nr Deussen Pk nr Houston TX

USGS 295554095093401 Lk Houston at mouth of Jack's Ditch nr Houston Tx

USGS 295724095092301 Lk Houston Site A nr Alco oil field nr Houston TX

USGS 295826095082200 Lk Houston S Union Pacific RR Bridge nr Houston TX

USGS 314515093064501 Sibley Lake about 825 ft north of spillway nr Natc Natchitoches Parish, Louisiana

USGS 314618093082101 Sibley Lake northwest branch about 0.5 miles south Natchitoches Parish, Louisiana

USGS 321328092293201 Caney Creek Reservoir about 850 ft west of spillwa Jackson Parish, Louisiana

USGS 322927093503701 Cross Lake near Ford Park beach at Shreveport LA

USGS 323035093475801 Cross Lake 300m W of spillway at Shreveport LA

USGS 323108093533901 Cross Lake near Bird Islands at Shreveport LA

USGS 323150091292701 Poverty Point Reservoir N beach nr Delhi LA

USGS 324242092204001 Lake D'Arbonne 300m W of dam nr Farmerville LA

USGS 324434092224101 Lake D'Arbonne 2.6mi E of Hwy33 nr Farmerville LA

USGS 324455092253401 Lake D'Arbonne nr Hwy 33 beach nr Farmerville LA

USGS 324531092253301 Lake D'Arbonne 0.5mi W of Hwy33 nr Farmerville LA

USGS 3505550820000 Municipal Reservoir 1 at River Oak Road

USGS 350624082035200 Lake Bowen below I-26(Site 5) SC

USGS 3506390815814 Municipal Reservoir No.1 at Dam nr. Fingerville SC

USGS 350641082014700 Lake Bowen below SC9(Site 10) near Fingerville SC

USGS 3506420820154 Mun Res 1 below Lake Bowen Dam nr Fingerville sc

USGS 390111084080300 Harsha Lake at East Fork State Park Beach OH

USGS 390121084054100 Harsha Lake at East Fork State Park Camp Beach OH

USGS 390433096534600 MILFORD LAKE OUTLET KS

USGS 390527096543400 MILFORD LAKE KS site 22

USGS 391008096550700 MILFORD LAKE KS site 19

USGS 391237096584800 MILFORD LAKE KS Site 15

USGS 391259097001800 MILFORD LAKE NEAR WAKEFIELD KS

USGS 393707083134100 Deer Creek Lake at Deer Creek State Park Beach OH

USGS 395431082310000 Buckeye Lake at Onion Island boat swim area OH

USGS 395520082281500 Buckeye Lake at State Park Fairfield Beach OH

USGS 395557082283800 Buckeye Lake at State Park Crystal Beach OH

USGS 395705083440100 CJ Brown Reservoir at Main Beach North OH

USGS 402003081095301 Tappan Lake at Scio upper I OH

USGS 403042084324100 Grand Lake Ohio west of Windy Point buoy B-2

USGS 403054084320500 Grand Lake Ohio east of Windy Point buoy B-3

USGS 403111084273200 Grand Lake Ohio county line south buoy B-6

USGS 403139084293900 Grand Lake Ohio central buoy B-1

USGS 403200084320000 Grand Lake Ohio north of Safety Island buoy B-4

USGS 403210084273300 Grand Lake Ohio county line north buoy B-5

USGS 405725081070600 Deer Creek Reservoir at Alliance I OH

USGS 412810082491000 Sandusky Bay at Driftwood Beach at Bay View OH

USGS 413051082561800 Lake Erie at Port Clinton I OH

USGS 413053082553000 Lake Erie at Lakeview Park at Port Clinton OH

USGS 413233082433700 Lake Erie at Marblehead I OH

USGS 413932082492300 Lake Erie at Put-in-Bay boat area OH

USGS 414023083171800 Lake Erie at Oregon I OH

USGS 414100083223600 Inland Lake Beach at Maumee Bay State Park OH

USGS 414111083223200 Maumee Bay at Maumee Bay State Park Cove 3 OH

USGS 414111083223201 Maumee Bay Coves 2-4 Composite at Maumee Bay S.P., OH

USGS 444232086073501 LOON LAKE NEAR BOAT LAUNCH AT SBDNL MI

USGS 444235086073701 LOON LAKE NR COVE AT SLEEPING BEAR DUNES NL MI

USGS 444235086074001 LOON LAKE NR PLATTE RIVER INLET AT SBDNL MI

USGS 444355086091101 LAKE MICHIGAN NEAR PLATTE POINT AT SBDNL MI

USGS 444546086043501 LAKE MICHIGAN NEAR ESCH ROAD AT SBDNL MI

USGS 445034086035701 NORTH BAR LAKE NEAR OUTLET AT SBDNL MI

USGS 450037086070801 FLORENCE LAKE NEAR BOAT LAUNCH AT SBDNL MI

USGS 462846086321301 MINERS LAKE NEAR SW SHORE AT PICTURED ROCKS MI

USGS 463516086185201 TRAPPERS LAKE NR CAMPGROUND AT PICTURED ROCKS MI

USGS 463835086015301 GRAND SABLE LAKE NR E SHORE AT PICTURED ROCKS mi

USGS 475814088582001 LAKE DESOR NR S DESOR CAMPGROUND AT ISLE ROYALE MI

USGS 480155088412101 LAKE RICHIE NR INDIAN PORTAGE TR AT ISLE ROYALE MI

USGS 480305088410601 LAKE RICHIE NR BEDROCK BEACH AT ISLE ROYALE MI

USGS 480308088471401 LAKE HARVEY NEAR NE OF LAKE AT ISLE ROYALE MI

USGS 480350088433001 CHICKENBONE LAKE NR E CB CAMPGROUND AT ISRO mi

USGS 480426088415401 CHICKENBONE LAKE NR W CB CAMPGROUND AT ISRO MI

USGS 480429088431001 CHICKENBONE LAKE NR OLD PORTAGE AT ISLE ROYALE

USGS 480557088391001 SARGENT LAKE NR NE END OF LAKE AT ISLE ROYALE MI

Supplementary table

Table B1

Variable	Total N (4388)	Maximum	Arithmetic mean	Median	Minimum
Longitude	4349	173.6	-73.9	-88.7	-124.6
Latitude	4348	60.4	38.5	40.4	-42.4
Secchi (m)	3037	36.7	1.8	1.1	0
pH	2573	10.5	8.1	8.2	2.8
Ammonium as nitrogen (mg/L)	2767	3.2	0.0	0.02	0
Nitrite as nitrogen (mg/L)	1827	0.4	0.0	0.0	0
Nitrate as nitrogen (mg/L)	3347	51.7	0.1	0.005	0
Nitrate + Nitrite as nitrogen (mg/L)	3271	52.1	0.1	0.005	0
Total Phosphorus (mg/L)	3562	11.0	0.1	0.04	0.001
Dissolved Organic Carbon (mg/L)	2763	515.8	8.2	5.3	0.2
Fluorometric Chlorophyll (µg/L)	3407	1500.0	26.7	8.3	0
Total nitrogen (mg/L)	3560	54.0	1.3	0.7	0.01
Turbidity NTU	2543	901.0	13.4	3.2	0.01
Specific Conductivity (µs/cm)	3542	64810.0	602.6	260.0	2.8
Microcystin (ug/L)	4388	440.0	2.9	0.1	0

Appendix C. (Chapter 3)

Supplementary tables and figures

Figure C1

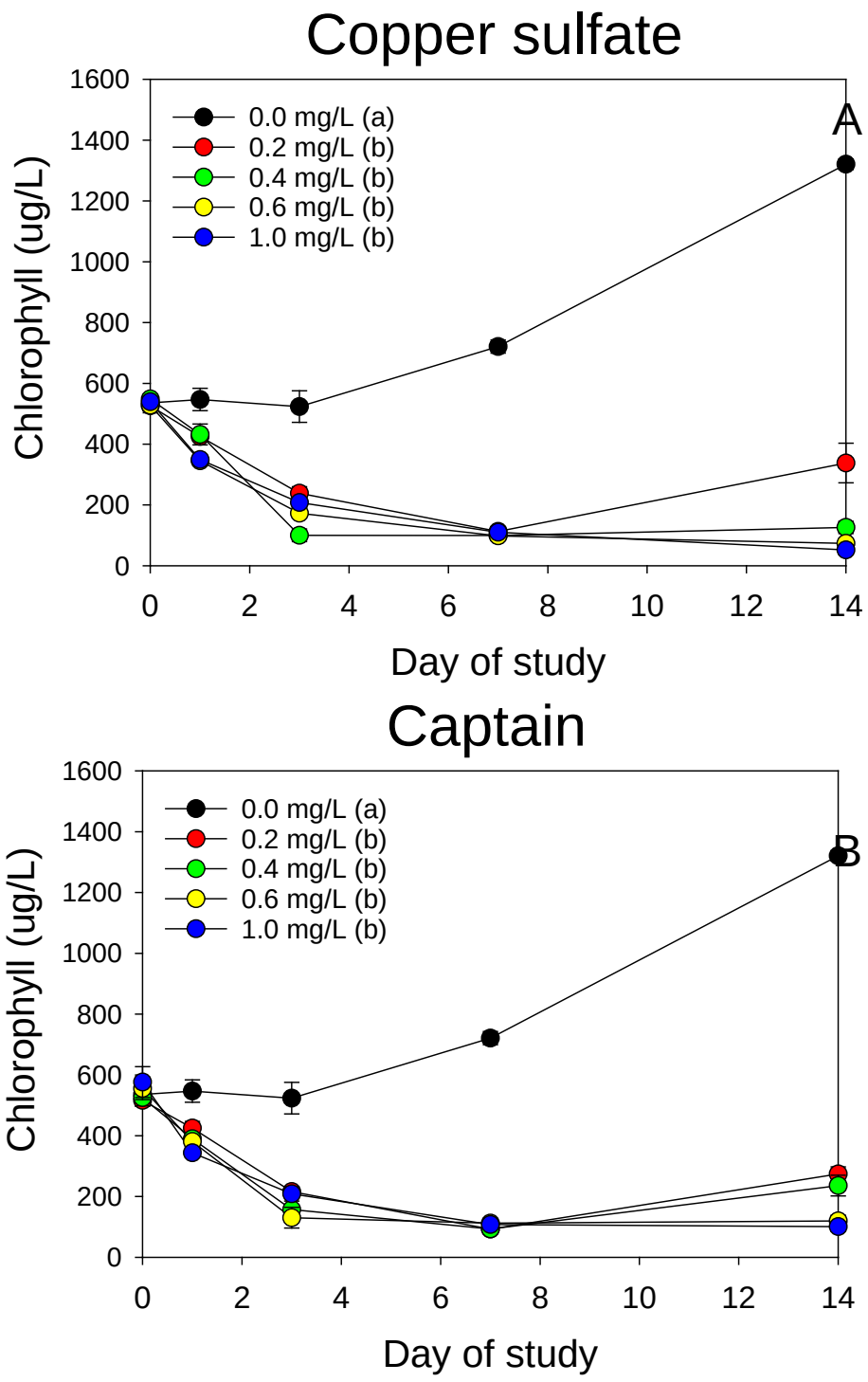

Figure C2

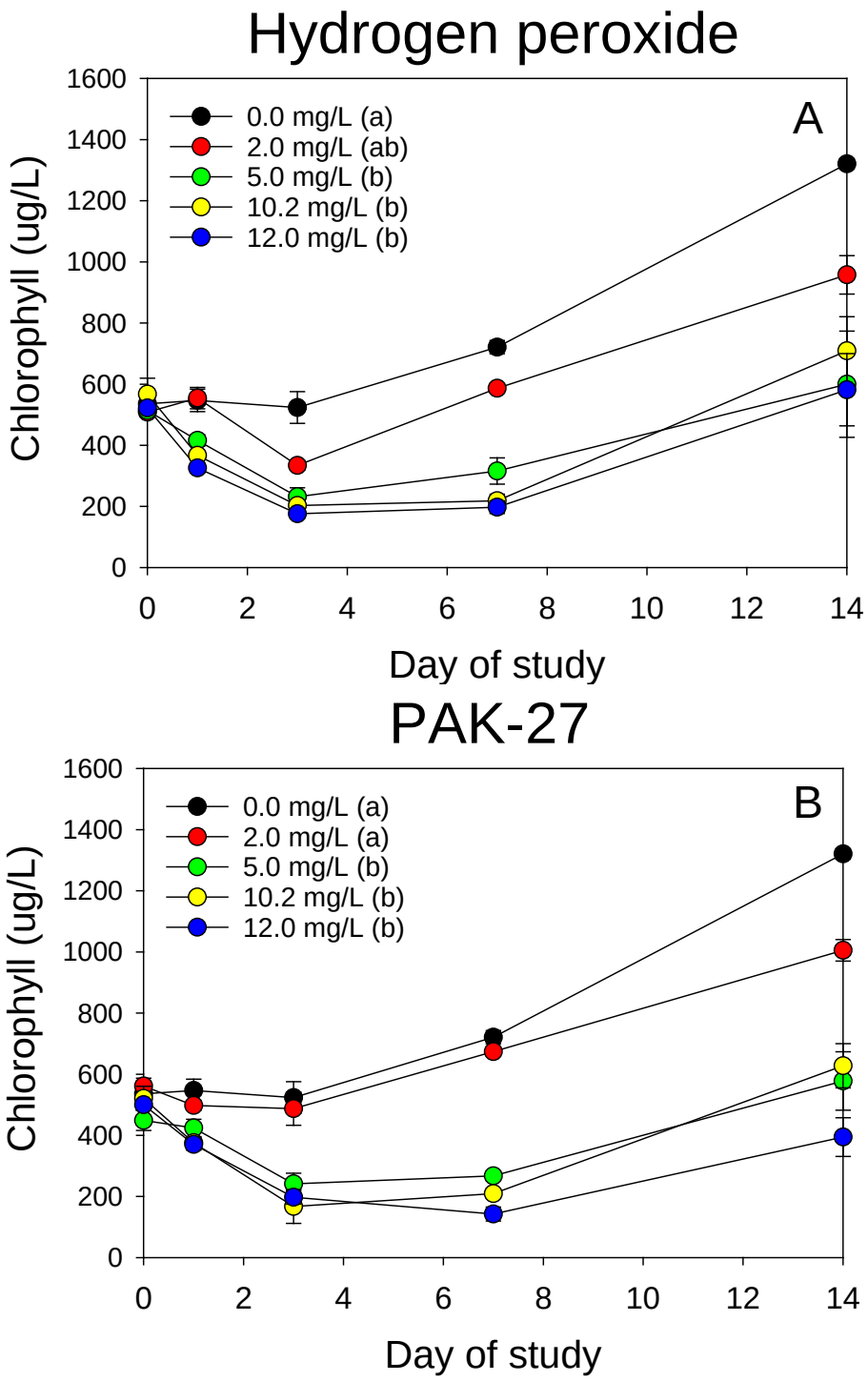
Hydrogen peroxide
A
0.0 mg/L (a)
2.0 mg/L (ab)
5.0 mg/L (b)
10.2 mg/L (b)
12.0 mg/L (b)
Chlorophyll (ug/L)
Day of study
PAK-27
B
0.0 mg/L (a)
2.0 mg/L (a)
5.0 mg/L (b)
10.2 mg/L (b)
12.0 mg/L (b)
Chlorophyll (ug/L)
Day of study

Figure C3

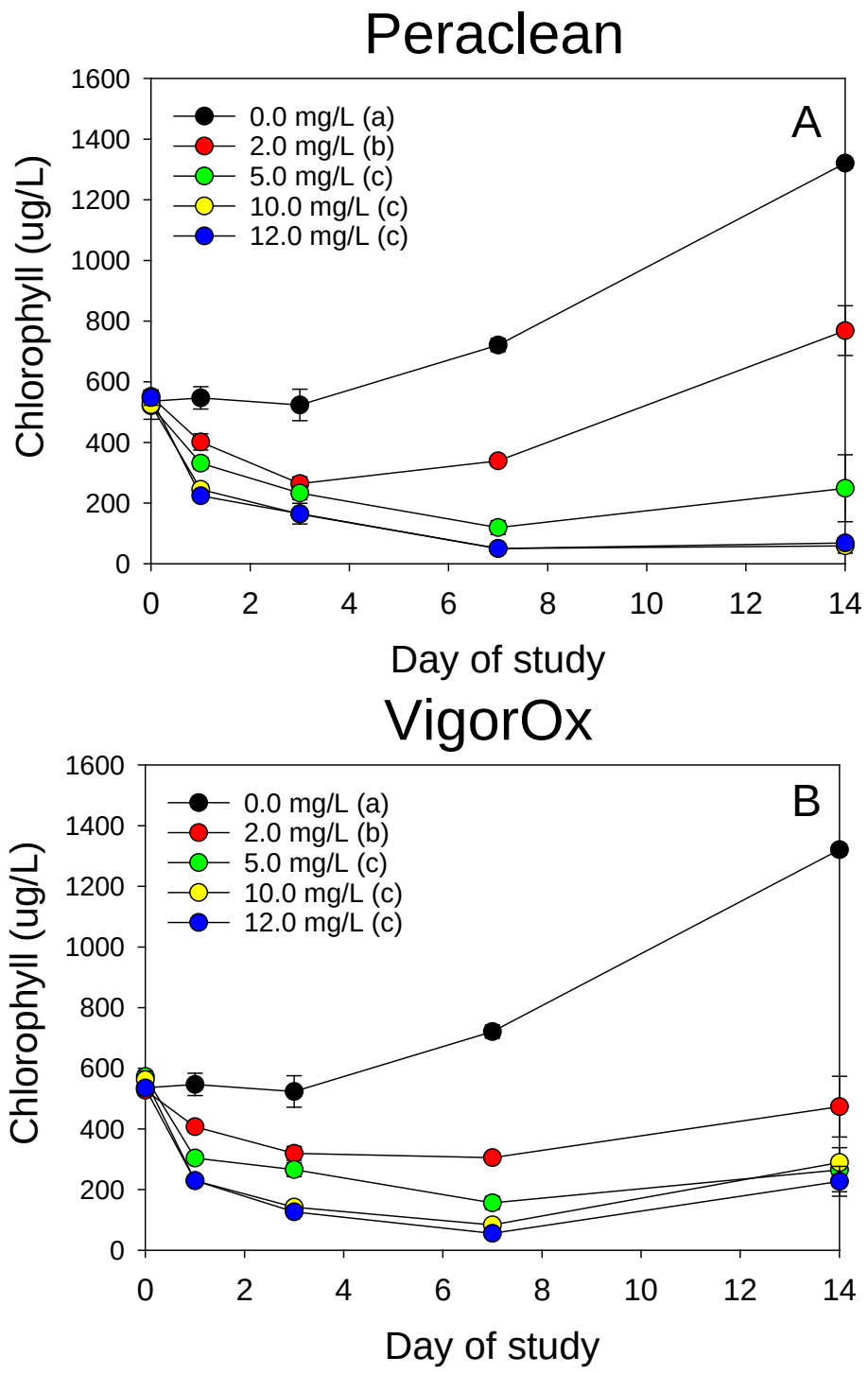
Peraclean
Chlorophyll (ug/L)
Day of study
0.0 mg/L (a)
2.0 mg/L (b)
5.0 mg/L (c)
10.0 mg/L (c)
12.0 mg/L (c)
A
VigorOx
B

Figure C4

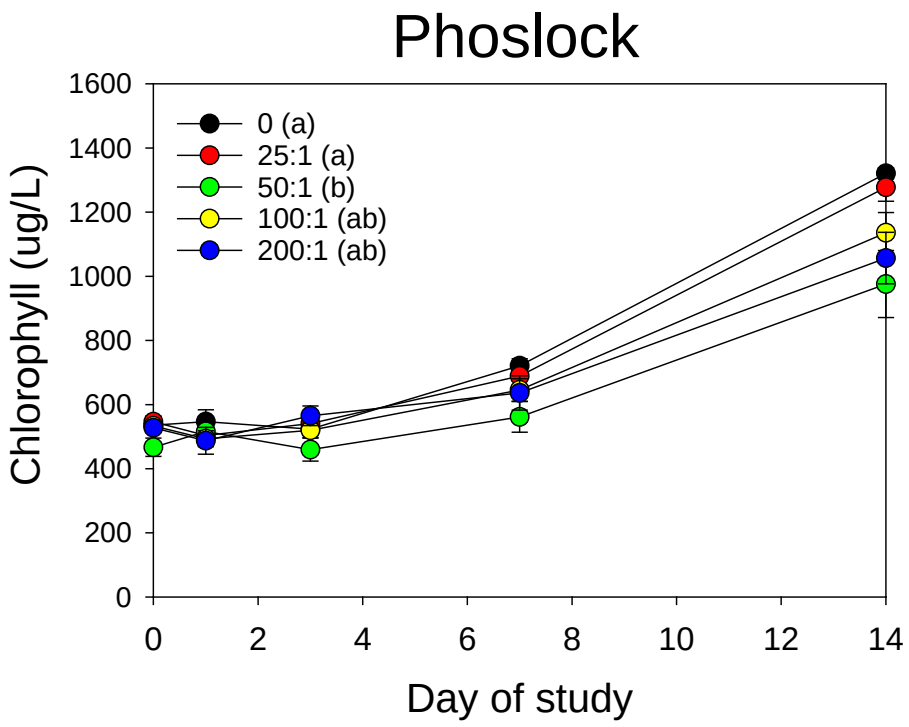
Phoslock
Chlorophyll (ug/L)
1600
1400
1200
1000
800
600
400
200
0
0 (a)
25:1 (a)
50:1 (b)
100:1 (ab)
200:1 (ab)
0
2
4
6
8
10
12
14
Day of study

Figure C5

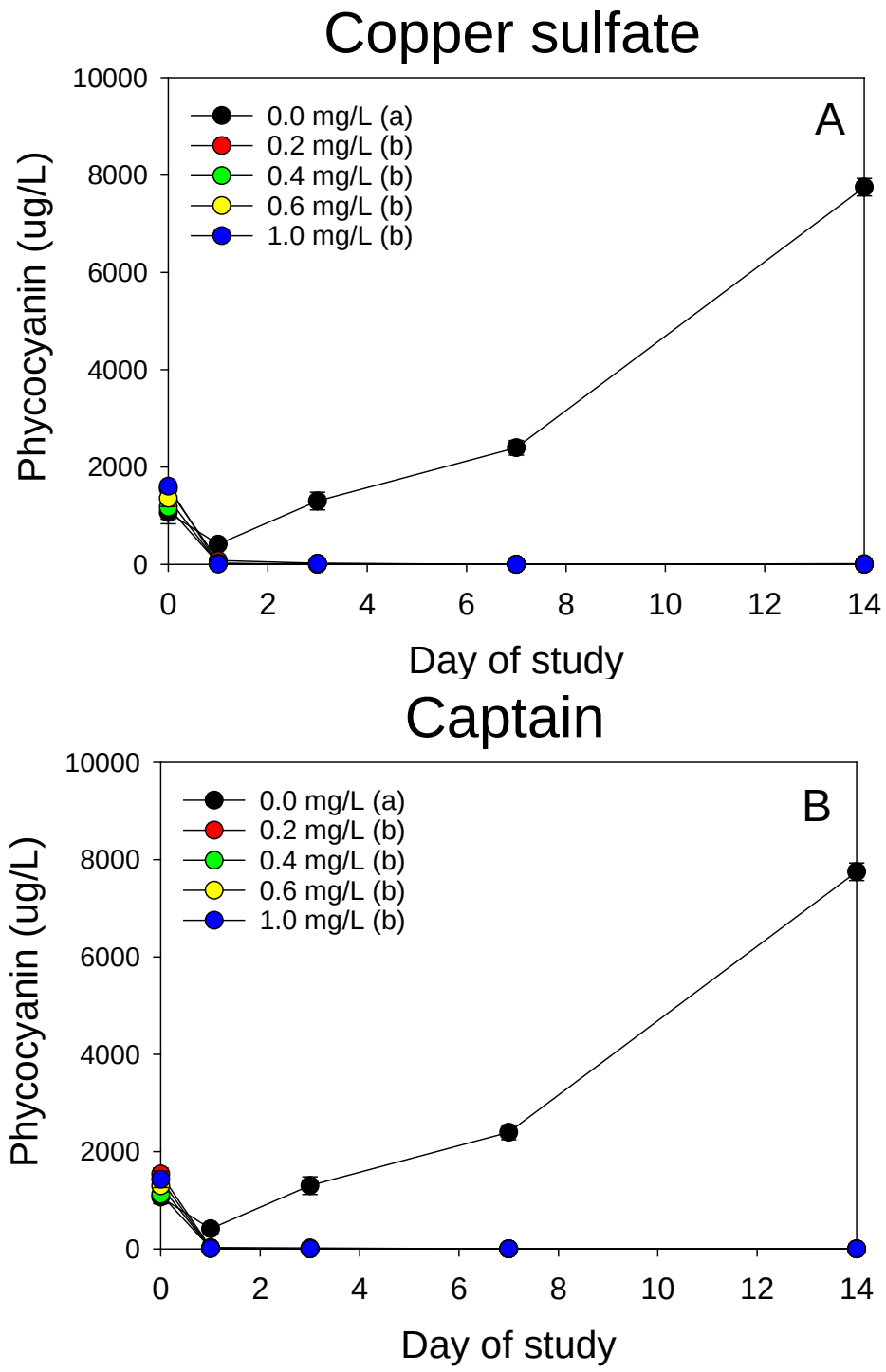
Copper sulfate
Phycocyanin (ug/L)
10000
8000
6000
4000
2000
0
0.0 mg/L (a)
0.2 mg/L (b)
0.4 mg/L (b)
0.6 mg/L (b)
1.0 mg/L (b)
A
0
2
4
6
8
10
12
14
Day of study
Captain
Phycocyanin (ug/L)
10000
8000
6000
4000
2000
0
0.0 mg/L (a)
0.2 mg/L (b)
0.4 mg/L (b)
0.6 mg/L (b)
1.0 mg/L (b)
B
0
2
4
6
8
10
12
14
Day of study

Figure C6

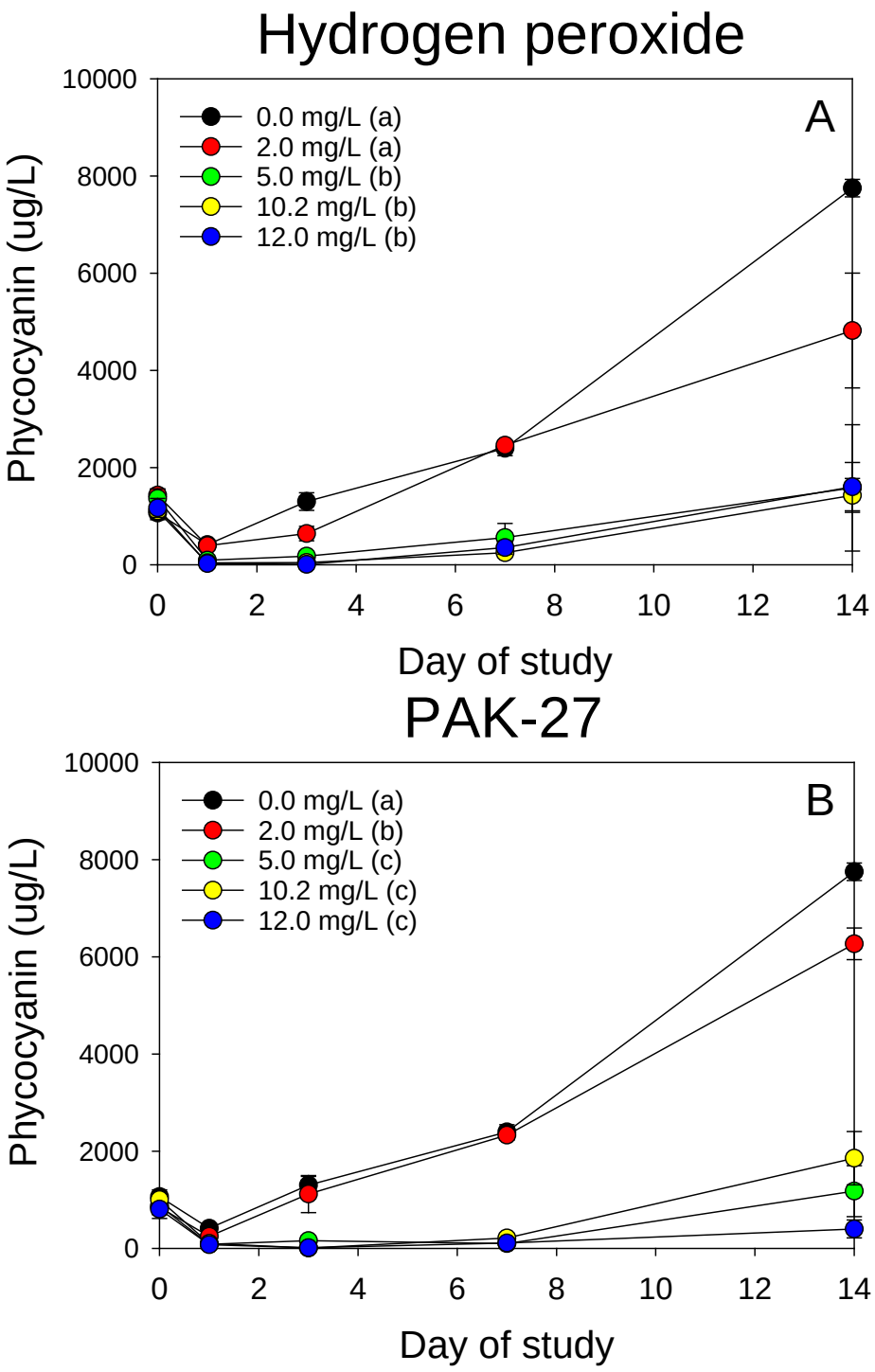
Hydrogen peroxide
A
0.0 mg/L (a)
2.0 mg/L (a)
5.0 mg/L (b)
10.2 mg/L (b)
12.0 mg/L (b)
Phycocyanin (ug/L)
Day of study
PAK-27
B
0.0 mg/L (a)
2.0 mg/L (b)
5.0 mg/L (c)
10.2 mg/L (c)
12.0 mg/L (c)
Phycocyanin (ug/L)
Day of study

Figure C7

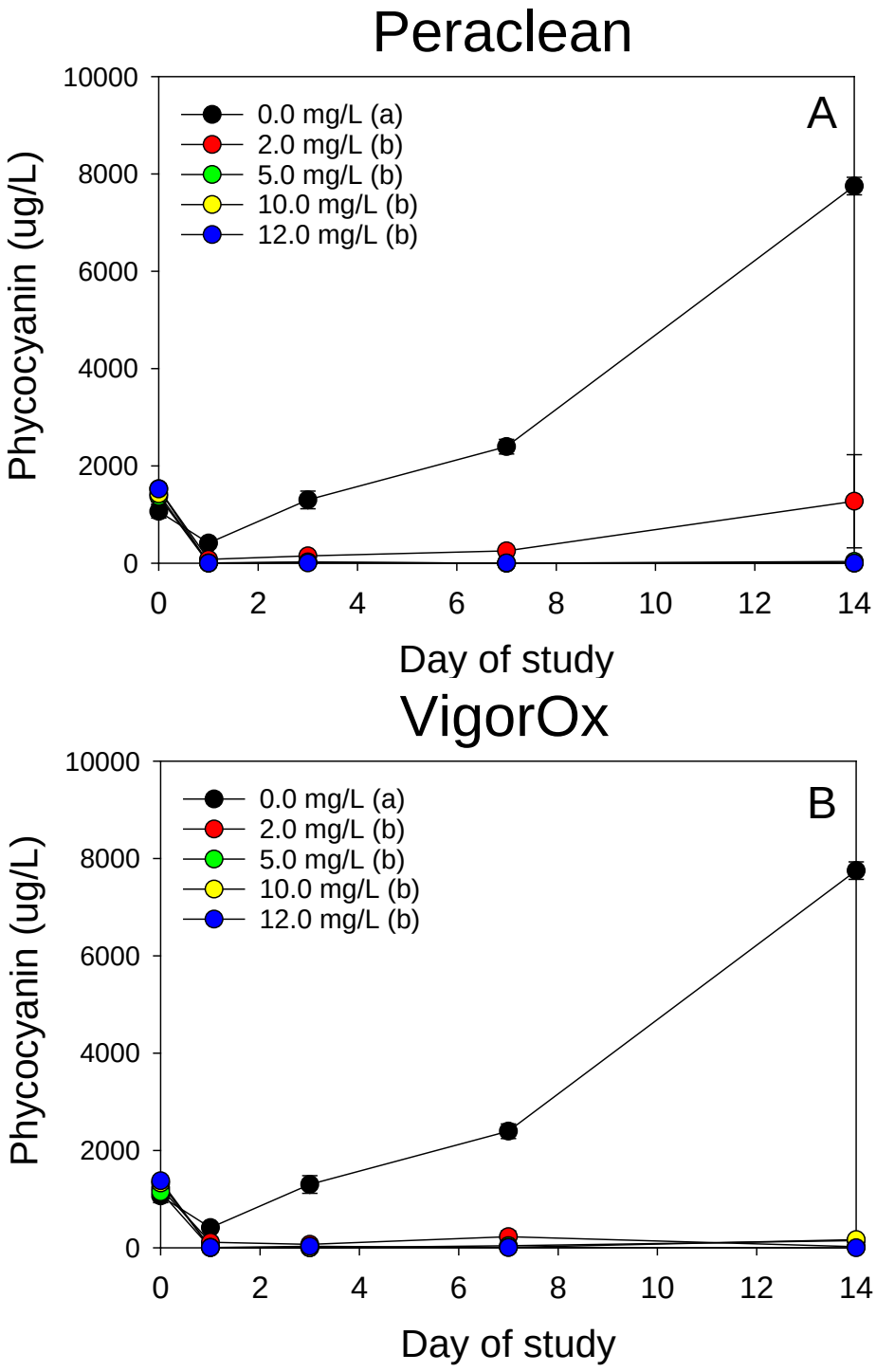
Peraclean
A
0.0 mg/L (a)
2.0 mg/L (b)
5.0 mg/L (b)
10.0 mg/L (b)
12.0 mg/L (b)
Phycocyanin (ug/L)
Day of study
VigorOx
B
0.0 mg/L (a)
2.0 mg/L (b)
5.0 mg/L (b)
10.0 mg/L (b)
12.0 mg/L (b)
Phycocyanin (ug/L)
Day of study

Figure C8

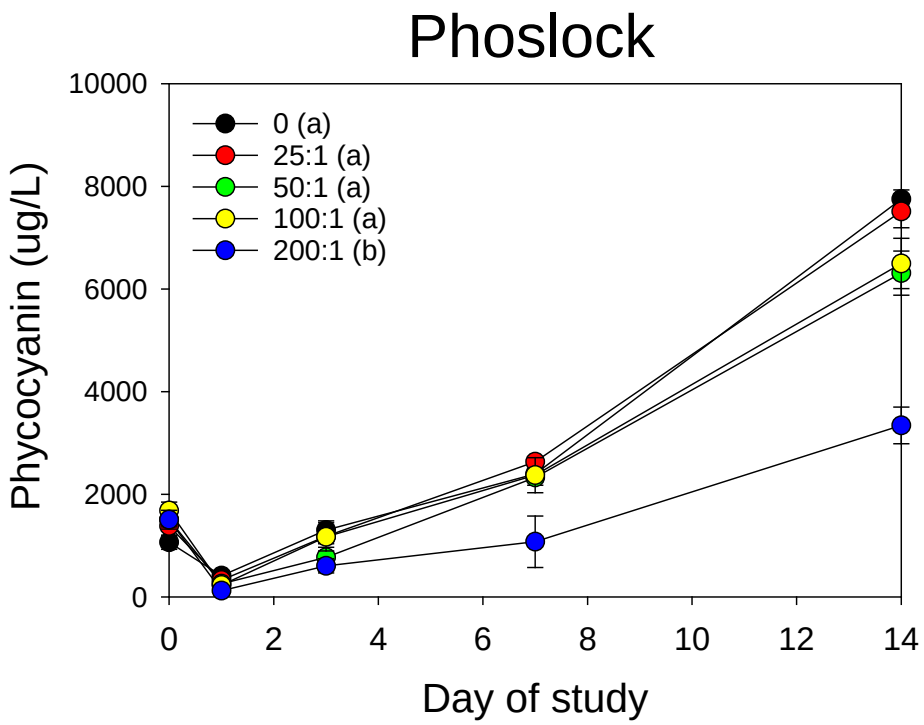
Phoslock
Phycocyanin (ug/L)
10000
8000
6000
4000
2000
0
0 (a)
25:1 (a)
50:1 (a)
100:1 (a)
200:1 (b)
0
2
4
6
8
10
12
14
Day of study

Table C1

Chemical	Copper sulfate	Captain	Hydrogen peroxide, liquid	PAK-27	VigorOx SP-15	Peraclean	Phoslock
Appearance	white/blue powder	blue liquid	colorless liquid	white powder	colorless liquid	colorless liquid	brown granules
Physical state	powder	liquid	liquid	solid	liquid	liquid	solid
Melting point (°C)	590	not available	-25	not reported	-49	-50	>1000
Boiling point (°C)	650	100	108	not applicable	109	>60	not applicable
Flash point (°C)	not applicable	>93.3	not applicable	not applicable	80	79	not applicable
Oxidizing properties	oxidizing	not available	oxidizing	oxidizing	oxidizing	oxidizing	not available
pH as solution	not determined	10-10.5	3.3	10.4-10.6	<1	-0.6	7-7.5