J. Aquat. Plant Manage. 57: 79–89

Sensitivity of *Microcystis aeruginosa* strains to copper and influence of phosphorus

WEST M. BISHOP, BEN E. WILLIS, AND ROBERT J. RICHARDSON*

ABSTRACT

Cyanobacterial blooms are widespread and increasingly affecting freshwater resources. Phosphorus (P) enrichment is often described as promoting blooms and may influence growth rates and response to management. The impact of P in growth media and cellular P content were assessed in terms of susceptibility of *Microcystis aeruginosa* (Kutzing) Lemmerman to copper sulfate. Five strains of *M. aeruginosa* were tested under three different P concentrations (1,500 µg L^{-1} = high; 150 µg L^{-1} = medium; 75 µg L^{-1} = low). All *M. aeruginosa* strains grown at low P concentration, compared with medium or high concentrations, had significantly decreased ($P < 0.05$) P and chlorophyll *a* content per cell, though strain 2664 still had significantly higher P content than the other strains. *Microcystis aeruginosa* strains grown in high and medium P concentrations had similar 96-h 50% lethal concentration (LC_{50}) on the basis of chlorophyll *a* content and cell densities. In the low-P growth concentration, *M. aeruginosa* strain 2386 had significantly decreased 96-h LC_{50} than all other *M. aeruginosa* strains, and *M. aeruginosa* 2665 had a significantly higher 96-h LC_{50} compared with *M. aeruginosa* strains 2386, 2388, and 2664. The relative sensitivities of strains grown in low-P medium to copper were *M. aeruginosa* 2386 > 2388 ≥ 2664 ≥ 2061 ≥ 2665. All strains had significantly decreased growth rates under low P compared with high P, but only *M. aeruginosa* 2386 had increased sensitivity to copper. This research provides insights about altered sensitivity of cyanobacteria to reduced P supplies. Decreasing P availability can decrease the amount and need for reactive copper algaecides by altering growth rates and carrying capacity of *M. aeruginosa* strains and, in specific cases, increase the sensitivity of cyanobacteria to copper.

Key words: algaecide, cyanobacteria, efficacy, nutrients.

INTRODUCTION

Copper has been widely used to manage nuisance algal assemblages in freshwaters (Oliveira-Filho et al. 2004). Management practices that decrease application of copper-based algaecides are needed to promote ecological safety and comply with regulatory standards (USEPA 2011). Methods that increase the relative sensitivity of a given algal strain to copper would be a valuable approach to decrease the amount of algaecide applied while also enhancing efficiency of use. With increasing eutrophication in freshwaters and corresponding harmful algal blooms, nutrient mitigation (e.g., decreasing loading or availability) should be a key focus of management (Hallegraeff 1993, USEPA 2007, Heisler et al. 2008). The implications of eutrophication may go beyond simply promoting the presence and growth of noxious algae. Previous research has shown that increased available aqueous phosphorus (P) in growth media has led to enhanced metal detoxification and decreased algal sensitivities to copper exposures (Rhee 1972, 1973, Jensen et al. 1982, Twiss and Nalewajko 1992, Guasch et al. 2004). Also, increases in cellular P content has coincided with a concomitant increase in copper tolerance, whereas P-limited cells were more susceptible (Wang and Dei 2006). By decreasing the external bioavailable P supply to algae in water, relative sensitivity to copper could be theoretically increased. In this research, the relationships between P bioavailability, cellular P content, and algal sensitivity were evaluated toward the goal of enhancing the efficiency of copper algaecide application.

Algae deficient in P have shown increased sensitivity to copper exposure compared with the same population grown under P-replete conditions (Twiss and Nalewajko 1992; Guasch et al. 2004; Rocha et al. 2016). This research investigates a mechanism that may account for the most significant component of an exposure (i.e., the organism), and how the inherent sensitivity can be shifted by limiting essential nutrients (Serra et al. 2010). Management strategies that remove bioavailable P from aqueous systems can be used to decrease cyanobacterial growth (van Oosterhout and Lürling 2013) and potentially increase sensitivities to applied copper algaecides. Relationships between aqueous P concentrations and copper toxicity have been measured in some laboratory (Twiss and Nalewajko 1992, Rocha et al. 2016) and field studies (Guasch et al. 2004) on sensitive green algae and mixed periphyton. Management of P concentrations may assist in indirect control of noxious cyanobacteria by limiting carrying capacity while promoting the growth of more beneficial algae (Smith 1983, Tilman et al. 1986, Seale et al. 1987). By shifting an algal population to a lower density/biomass, a concomitant decreased amount of copper would be required to achieve a critical burden threshold (i.e., amount of copper per unit biomass needed for control; Bishop and Rodgers 2012). This study provides water resource managers with helpful information regarding the efficiency of managing noxious algal infestations by integrating P mitigation with algaecide controls.

Phosphorus can be important in harmful cyanobacterial dominance (Rhee 1982, Tilman et al. 1982); therefore,

*First and second authors: Algae Scientist & Water Quality Research Manager and Aquatic Development Scientist, SePRO Research and Technology Campus, 16013 Watson Seed Farm Rd. Whitakers, NC 27891. Third author: Professor and Extension Specialist, Department of Crop and Soil Sciences, 4401B Williams Hall, North Carolina State University Raleigh, NC 27695-7620. Corresponding author's e-mail: westb@sepro.com. Received for publication October 15, 2018, and in revised form January 13, 2019.

decreasing P may be a strategic approach to managing nuisance algal infestations (Grantz et al. 2013, Bishop and Richardson 2018). Inherent sensitivities differ among algal species and strains, so species-specific shifts in response to P mitigation should also differ (Hutchinson and Stokes 1975, Wu et al. 2017). Differences in nutrient use, salinity/stress tolerance, morphological characteristics (Lakeman et al. 2009), and copper sensitivity (Twiss et al. 1993, Garcia-Villada et al. 2004) have been documented for different algal strains. Alteration of P concentrations was specifically evaluated in this study, as it is expected to affect noxious cyanobacteria to a greater extent than other algal types (Downing et al. 2001, Saxton et al. 2012).

The cyanobacterium *Microcystis aeruginosa* (Kutzing) Lemmerman is widespread globally in freshwaters (Reynolds and Walsby 1975, Chorus and Bartram 1999). *Microcystis aeruginosa* can be a prolific toxin producer and can have severe impacts on the quality of potable water, impede recreational activities, and kill livestock (Carmichael 2001). Negative ecological impacts are commonly observed during and after *M. aeruginosa* blooms, such as hypoxic zones (Paerl 1988), depressed fisheries health (Zhao et al. 2012, Liu et al. 2014) and impaired food-web integrity (Lampert 1982). *Microcystis aeruginosa* has numerous adaptations to low-P environments, including storage (Jacobson and Halmann 1982, Whitton et al. 1991), luxury uptake (Baldia et al. 2007), use of high-affinity transporters (Harke et al. 2012), alkaline phosphatase activity (Štrojsová et al. 2005), and upregulation of multiple genes in P-limiting conditions (Harke et al. 2012). Buoyancy regulation in *M. aeruginosa* allows access to the hypolimnion to sequester P (Kromkamp et al. 1989, Jacoby and Frazer 2009), especially when epilimnetic P is limiting (Ganf and Oliver 1982). *Microcystis aeruginosa* can also acquire and use diverse forms of P (Štrojsová et al. 2005, Saxton et al. 2011). The majority of P accumulated in *M. aeruginosa* is typically internalized (89%; Saxton et al. 2012) and stored as polyphosphate bodies that can be used to maintain growth in P-limiting conditions (Reynolds 2006, Shen and Song 2007). Polyphosphate bodies have a high affinity for metals, and can sequester and ameliorate toxicity of cadmium, copper, lead, zinc (Twiss and Nalewajko 1992, Verma et al. 1993), and iron (Chaffin et al. 2011). More data are needed to evaluate the influence of P concentrations and accumulated P content on *M. aeruginosa* susceptibility to various management practices.

Growth rates and algal health often parallel essential nutrient availability (Watson et al. 1997). By evaluating the increase in sensitivity to altered P concentrations, more efficient management can be implemented. P concentrations in streams and lakes have been increasing over the past decades (Stoddard et al. 2016). The low and medium P concentrations tested in this research are in line with eutrophic and hypereutrophic conditions that have been commonly found in disturbed lakes (USEPA 2016). The medium and high P concentrations tested are also representative of agricultural runoff (Hart et al. 2004) and storm-water runoff (Yang and Lusk 2018), as well as sediment release (Søndergaard et al. 2001). Management strategies can be created that incorporate multiple approaches (e.g., P mitigation and copper exposures) to achieve desired control. The overall goals of this research were to measure sensitivity of different *M. aeruginosa* strains to copper exposures and assess changes in sensitivities in response to altered P concentrations. This research can be used to increase the efficiency of reactive algaecide programs while decreasing routine dependence on algaecide use and the overall algaecide loads applied.

The specific objectives of this research were to: 1) measure the sensitivities of five cultured *M. aeruginosa* strains to copper in different P concentrations and 2) compare the relative sensitivities of *M. aeruginosa* strains and assess the influence of cellular P content.

MATERIALS AND METHODS

Strains and exposure-testing concentrations

Five strains of *M. aeruginosa* were obtained from the University of Texas at Austin culture collection (Table 1). Testing commenced with strains maintained in a stable, healthy culture within 3 mo of culture initiation. They were cultured and tested in ultrafiltration-purified COMBO nutrient media (Kilham et al. 1998; Table 2). Cultures were maintained in a controlled environment at 23 C ± 1 C and a 16-h light/8-h dark photoperiod with fluorescent lighting (Spectralux T5/HO 6500K blue; 3000K red) at an intensity of 67.5 ± 2.7 μmol photons m^{-2} s (Lewis et al. 1994). Total microcystins (both intra- and extracellular) were measured after three freeze/thaw cycles in the dark in recently initiated cultures and cultures maintained for > 5 mo using a microcystins/nodularins enzyme-linked immunosorbent assay kit[1]. The microcystin-LR variant was used as the calibrator, though results are congener independent as the kit tests for the ADDA side chain consistent among all microcystin variants.

Experiments compared different initial concentrations of bioavailable, free P (on the basis of decreased supplies of dipotassium phosphate in the media) with algal growth rates, chlorophyll *a* content, and cell densities through time. Cell densities, chlorophyll *a* content, and P concentration were measured initially and 96 h after exposure to copper sulfate pentahydrate[2] ($CuSO_4 \cdot 5\ H_2O$; C489-1 Fisher Scientific, Inc.) amendments, including 0.031, 0.0625, 0.125, 0.25, or 0.5 mg Cu L^{-1} in treatments ($n = 3$; 125-ml volume in 150-ml acid-washed Erlenmeyer flasks). Controls did not receive copper amendments. Experiments were replicated in time and results pooled for analysis. Aqueous total copper concentrations were measured by taking 15 ml of exposure solution, acidifying to 2% v v^{-1} with trace-metal-grade nitric acid,[3] then filtering (0.22-μm Whatman GF/F glass microfiber[4]). Copper was measured using inductively coupled plasma–optical emission spectrometry[5] (ICPE 9000; Shimadzu Corporation), including a matrix-matched calibration curve from serial dilution of a 1,000 mg Cu L^{-1} standard[6] (Fisher Scientific, Inc. SC194; SMEWW 2005). The limit of detection for copper was 1 μg L^{-1}. Method blanks were analyzed with each run to ensure no contamination by the materials used in sample preparation or analysis.

Phosphorus growth concentration

The cyanobacteria were grown in three different P concentrations to assess the effect of P supply on growth

Table. 1. Description of the five *Microcystis aeruginosa* strains used in testing and average growth rates throughout exposure duration. Cell diameter was measured at experiment initiation and conclusion.

Description	Strain 2386	Strain 2388	Strain 2664	Strain 2061	Strain 2665
Isolation number	NRC-1(ss-17)	s-15-b	UWOCC019	Patterson's 1036 AX	UWOCC 017
Origin	Little Rideau Lake, Ontario, Canada	Bruno, Saskatchewa, Canada	W.P. Game Reserve, Winburg, South Africa	Lake Mendota, Madison, WI	Rietvlei Dam, Pretoria, South Africa
Cell diameter, μm	3.0–5.5	3.5–6.0	6.0–8.5	1.0–3.0	4.3–6.5
Toxin producer	No	Possible	Yes	Possible	Yes
Growth rate, d^{-1} (high P)	0.154	0.101	0.062	0.245	0.193
Growth rate, d^{-1} (medium P)	0.130	0.069	0.058	0.169	0.089
Growth rate, d^{-1} (low P)	0.009	0.027	0.036	0.036	0.012

rates and sensitivities. Treatments of COMBO media P concentrations were 1,500 ± 90 μg P L^{-1} for high media (high P), medium P was 150 ± 20 μg P L^{-1}, and 75 ± 10 μg P L^{-1} represented the low-P treatment concentration. Growth rates differed among treatments and strains. *Microcystis aeruginosa* densities of 5×10^6 cells ml^{-1} were used for initiation of toxicity experiments. *Microcystis aeruginosa* grown in high and medium P concentrations were mostly in exponential growth phase when cell densities attained testing levels, whereas the cultures were approaching stationary phase in the low-P concentration medium (Table 1). Growth phase of cultures was based on relation to carrying capacity. Phenotypes and densities of *M. aeruginosa* can alter exposure to copper (Franklin et al. 2002, Wu et al. 2007), so similar densities of cells and unicellular or dividing-phase cells for testing, and no large (> 10 cell) colonies were observed, as this could have altered the exposure efficiency.

Algal response parameters

Responses were measured initially and 96-h post-treatment and toxicity compared on the basis of the percent inhibition from untreated controls. The growth rate, μ, was calculated as follows:

$$\mu = \ln(N_t N_0^{-1}) t^{-1}. \quad [1]$$

N_t and N_0 represent final and initial cell densities respectively, and t is the exposure time after test initiation. Doubling time (T_t) was calculated from growth rate by:

$$T_t = 0.6931 \mu^{-1}. \quad [2]$$

Percent inhibition of growth (as cell density and chlorophyll *a* concentration) for each treatment was calculated as follows:

$$\%\text{Inhibition} = ([\mu_c - \mu_t]\mu_c^{-1}) \times 100. \quad [3]$$

μ_c and μ_t denote the mean value of response in the control and treatments, respectively. Homogenized subsamples (10 ml) of *M. aeruginosa* treatments were filtered (0.45-μm nitrocellulose) and used to measure chlorophyll *a* concentration. Each sample was placed in 5 ml of buffered acetone and sonicated to lyse cells (modified Standard Methods for the Examination of Water and Watewater [SMEWW] 2005). Chlorophyll *a* was measured fluorometrically using a Wallac Victor[2] spectrofluorometer[7] by correlating with a matrix-matched standard calibration curve (10 to 640 μg L^{-1}; Sigma C-5753). Spectrophotometric scan (390 to 700 nm) was used to ensure chlorophyll *a* was the dominant light-harvesting pigment. Cell viability was measured by adding the mortal stain, methylene blue,[8] to homogenized subsamples from *M. aeruginosa* exposure vessels, targeting a final concentration of 0.02%. Counts of viable cells (not stained blue; Corradi and Gorbi 1993) under a Zeiss Axioskop 20 light microscope[9] were conducted after 5 min of exposure. Cell densities were measured using an improved Neubauer hemocytometer[10] (Hausser Scientific Co.) according to standard methods (10200 E/F, SMEWW 2005).

Phosphorus content

Phosphorus content per cell was evaluated from 10-ml samples of homogenized cultures immediately before the experiments. Two techniques were used to confirm consistent results. First, cells were centrifuged at 1,970 × *g* for 10

Table 2. Mean and ranges of water chemistry parameters for COMBO nutrient media used throughout all replicate experimentation.

Water sample parameter	Measurement (Range)	Measurement (Mean)
pH	7.4–8.5	8.2
Alkalinity (mg L^{-1} as calcium carbonate [$CaCO_3$])	15.6–19.8	18.1
Hardness (mg L^{-1} as $CaCO_3$)	38.1–45.4	42.2
Conductivity (μS cm^{-1})	230–440	318
Dissolved oxygen (mg L^{-1})	8.1–9.2	8.5
Total suspended Solids (mg L^{-1})	< 0.1	< 0.1
Chlorophyll *a* (μg L^{-1})	< 1	< 1
Total phosphorus (μg L^{-1})	1,410–1,590 (high) 130–170 (medium) 65–85 (low)	1,480 (high) 154 (medium) 73 (low)
Total Kjeldahl nitrogen (mg L^{-1})	0.22–0.25	0.23
Nitrate and nitrite (mg L^{-1})	5–13	7.4
Turbidity (nephelometric turbidity units)	< 1	< 1

min and the pellet was analyzed. Second, 10 ml of homogenized sample (5×10^6 cells ml^{-1}) was gently filtered through a 0.45-µm nitrocellulose filter, rinsed with 10 ml of COMBO media (–P) to remove any loosely adsorbed P, and then the cells and filtrate were analyzed separately for P. Results were similar between methods (5% variation), and the filtration technique was selected for use to account for loosely associated external P. Total P in cells and in water was analyzed after persulfate digestion (10 ml, 11 N sulfuric acid[11]) and analysis on a discrete analyzer[12] (Konelab Aqua 420) according to standard methods (USEPA 1978). Calibration was conducted using P reference standard[13] (Thermofisher Scientific, Inc.) of known concentration, processed in the same manner as sample materials. An estimate of P content per cell was calculated by dividing the mass of P attained by the number of cells filtered ($50 \pm 0.5 \times 10^6$ cells). All essential nutrients were supplied in excess in this experiment to decrease any confounding factors from colimitation, especially as found with iron (Nagai et al. 2007, Sterner 2008, Chaffin et al. 2011).

Statistics

A one-way ANOVA and Dunnett's procedure were used to assess significant differences between untreated controls and P and algaecide treatments. ANOVA and Tukey's multiple comparison procedure ($\alpha = 0.05$) were used to compare responses (growth rate, P content, chlorophyll *a* content, 50% lethal concentration [LC_{50}] value) in different P growth concentrations (McDonald 2014). Differences in sensitivities between strains and between P concentrations within strains were also compared. Nonlinear regression, using a polynomial quadratic equation ($y = y_0 + ax + bx^2$), best fit the data and was used to assess the exposure-response relationship and to calculate the 96-h LC_{50} values of copper for each strain at each P treatment concentration. A Shapiro-Wilk test was used to assess normality of the data, and all data were tested for constant variance (*F* test; McDonald 2014). All data were analyzed using Microsoft Excel[14] and SigmaPlot version 12.5.[15]

RESULTS AND DISCUSSION

Phosphorus concentration and *M. aeruginosa*

Elevated P concentration in the media consistently led to elevated P content in *M. aeruginosa* cells. There was a wide range in average P content of cells across all *M. aeruginosa* strains tested (19 to 238 fg P $cell^{-1}$), as has been reported in other works (e.g., 39 to 173 fg P $cell^{-1}$; Saxton et al. 2012). Saxton et al. (2012) also found significant variance in *M. aeruginosa* strains in terms of the cellular P content in relation to aqueous P concentration. Though differences in P content among strains were found at a given P concentration, each strain had significantly increased P content with each increase in P concentration in the media (Figure 1). The P concentrations tested in this research are representative of eutrophic and hypereutrophic systems that have been increasingly documented (USEPA 2016) and also of high P concentrations found in runoff or lakes subject to internal release (Søndergaard et al. 2001, Hart et al. 2004).

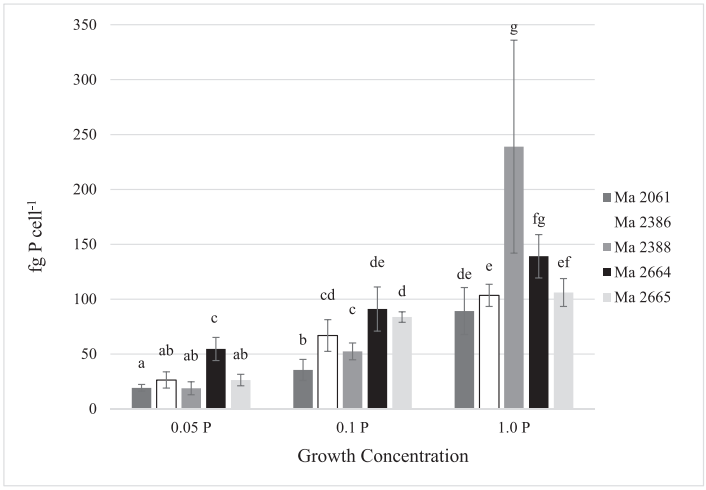

Figure 1. Mean P content per cell for five cultured *Microcystis aeruginosa* strains across three P growth concentrations. Error bars represent 1 standard deviation from the mean. Different letters signify significant differences ($P < 0.05$).

The cellular chlorophyll *a* content is species and strain specific (Rhee and Gotham 1981). Here, increased chlorophyll *a* content was measured with increases in P concentrations. *Microcystis aeruginosa* strain 2664 had similar chlorophyll *a* content in the low and medium P concentrations, although chlorophyll *a* content in the high P concentration was significantly higher than in the low-P treatment ($P < 0.05$). All other *M. aeruginosa* strains had significantly higher chlorophyll *a* content with each increase in aqueous P concentration (Figure 2).

Growth rates in the low-P media were significantly lower than in the high-P media for all strains and ranged from 0.009 d^{-1} (doubling time 77 d^{-1}) in *M. aeruginosa* 2386 to 0.036 d^{-1} (doubling time 19 d^{-1}) for *M. aeruginosa* 2664 and *M. aeruginosa* 2061 ($P < 0.05$; Table 1). Growth rates in the high-P and medium-P concentrations ranged from 0.058 to 0.245 d^{-1} for all strains (doubling time 4 to 12 d^{-1}) and were similar to those found for two different *M. aeruginosa* strains in previous research (0.058 to 0.162 d^{-1}; Saxton et al. 2012).

Sensitivities of *M. aeruginosa* strains

Since the growth rates and chlorophyll *a* content of *M. aeruginosa* differed among strains and P concentrations, the level of response that encompassed the 96-h LC_{50} and potency slopes also differed (Figures 3–8). At the high-P and medium-P concentrations, all *M. aeruginosa* strains tested had similar 96-h LC_{50} values. These ranged from 0.068 to 0.139 mg Cu L^{-1} on the basis of chlorophyll *a* content, and 0.047 to 0.168 mg Cu L^{-1} on the basis of cell densities (Figures 9 and 10; Table 3). In the low-P growth concentration, *M. aeruginosa* strain 2386 had a significantly lower 96-h LC_{50} value, on the basis of chlorophyll *a* content and cell density, than all other *M. aeruginosa* strains. Additionally, *M. aeruginosa* strain 2665 had a significantly higher 96-h LC_{50} value than *M. aeruginosa* strains 2386, 2388, or 2664 (Figure 3;

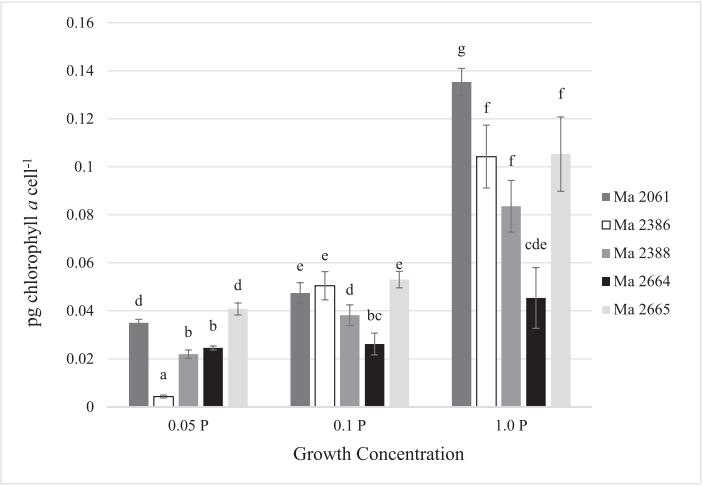

Figure 2. Mean chlorophyll *a* content per cell for five cultured *Microcystis aeruginosa* strains across three P growth concentrations. Error bars represent 1 standard deviation from the mean. Different letters signify significant differences ($P < 0.05$).

Table 3). The relative sensitivities of strains grown in low-P medium to copper were *M. aeruginosa* 2386 > 2388 ≥ 2664 ≥ 2061 ≥ 2665.

Toxicological influence of P content

The cyanobacterium *M. aeruginosa* has multiple adaptations for acquiring P, including acquisition of diverse forms of P (Štrojsová et al. 2005, Saxton et al. 2011) and access to P supplies in lake sediments (Barbiero and Welch 1992, Jacoby and Frazer 2009). Harke and Gobler (2013) reported that

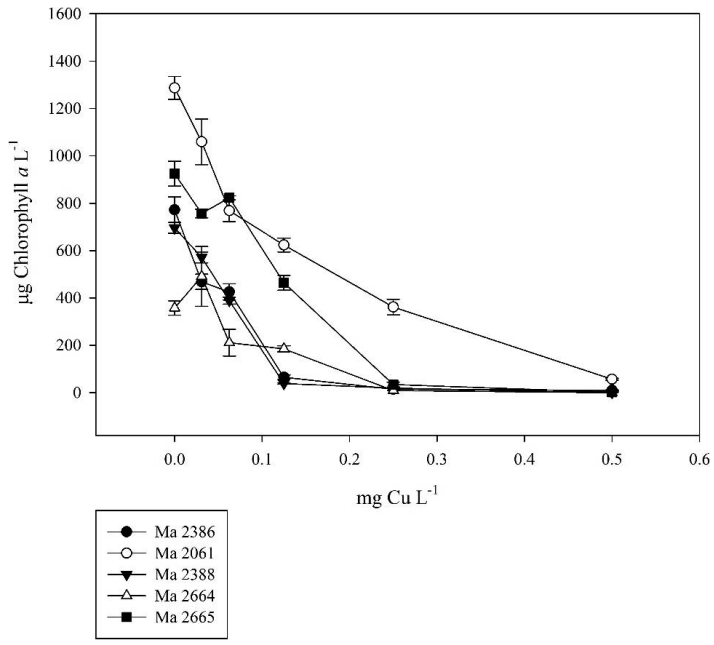

Figure 3. Mean chlorophyll *a* content of five *Microcystis aeruginosa* strains grown in high-P media after a 96-h exposure to copper sulfate pentahydrate. Error bars represent 1 standard error from the mean.

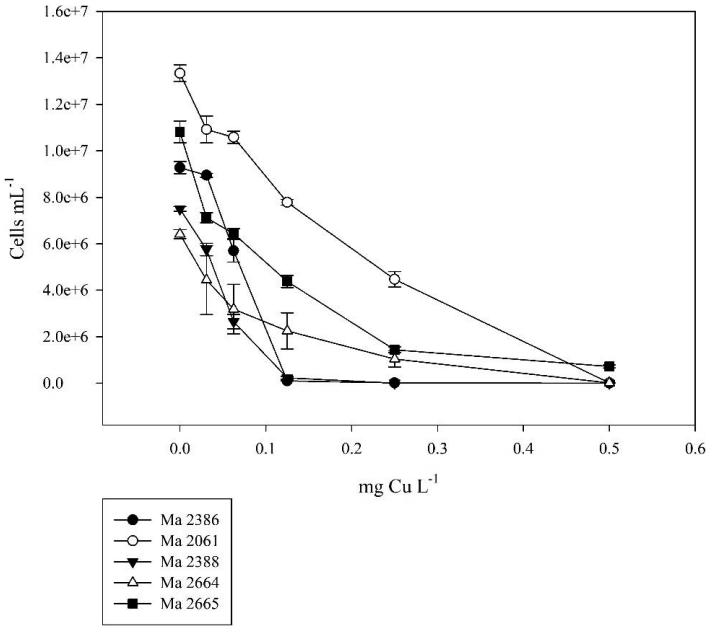

Figure 4. Mean cell densities of five *Microcystis aeruginosa* strains grown in high-P media after a 96-h exposure to copper sulfate pentahydrate. Error bars represent 1 standard error from the mean.

much of the *M. aeruginosa* genome was differentially expressed under P limitation, which may trigger stress response genes that could alter susceptibility. Here, cyanobacteria were cultured in a consistent P media to minimize impacts from P storage or altered P acclimation conditions (as in Rocha et al. 2016). Under chronic decreased P concentrations, alterations in cellular processes have been measured, including decreased protein content (Ji and Sherrell 2008), cell division processes, chlorophyll *a* synthesis, and photosynthetic rate (Van Mooy et al. 2009). Low P can also promote increased cell membrane permeability because of a decrease in phospholipids, which may allow for increased penetration of copper (Lombardi and Wangersky 1991). Although chlorophyll *a* content was significantly related to P concentrations across all strains, only one strain (*M. aeruginosa* 2386) had significantly increased sensitivity to copper.

P content and copper sensitivity

Copper is often used to control cyanobacterial blooms (Hrudey et al 1999). This action can provide immediate relief, albeit temporary control (Haughey et al. 2000, Mastin et al. 2002). Understanding the amount of copper needed to efficiently control the target population is important for decreasing the copper applied while restoring the functionality of the water resource (Mastin and Rodgers 2000). Decreasing copper use can offset potential impacts of repeated copper use such as altering zooplankton assemblage (Jacob et al. 2016), increasing sediment copper concentrations (Han et al. 2001), and sublethal impacts to sensitive fish species (Hansen et al. 1999). Toxic modes of action of copper are mostly internal to the algal cell, including free-radical production and inhibition of photo-

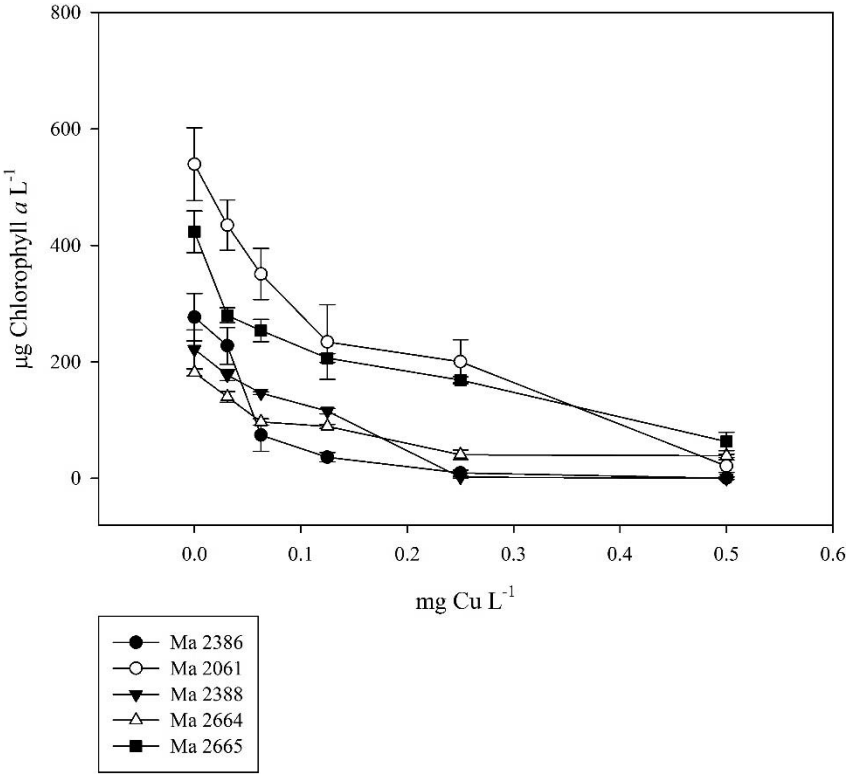

Figure 5. Mean chlorophyll *a* content of five *Microcystis aeruginosa* strains grown in medium-P media after a 96-h copper exposure to copper sulfate pentahydrate. Error bars represent 1 standard error from the mean.

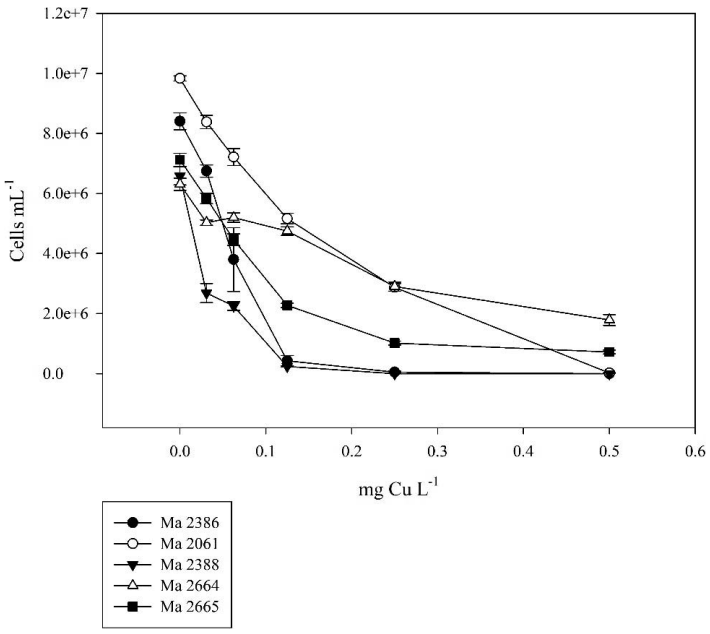

Figure 6. Mean cell densities of five *Microcystis aeruginosa* strains grown in medium-P media after a 96-h exposure to copper sulfate pentahydrate. Error bars represent 1 standard error from the mean.

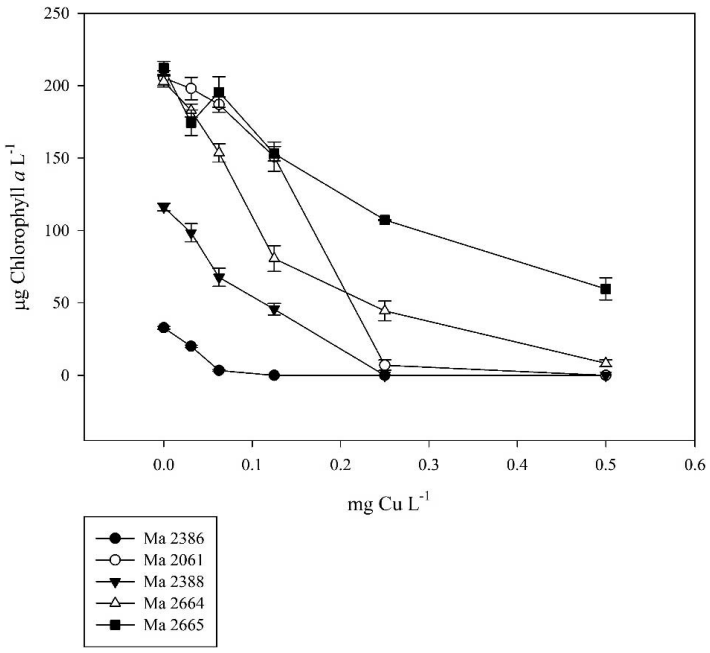

Figure 7. Mean chlorophyll *a* content of five *Microcystis aeruginosa* strains grown in low-P media after a 96-h exposure to copper sulfate pentahydrate. Error bars represent 1 standard error from the mean.

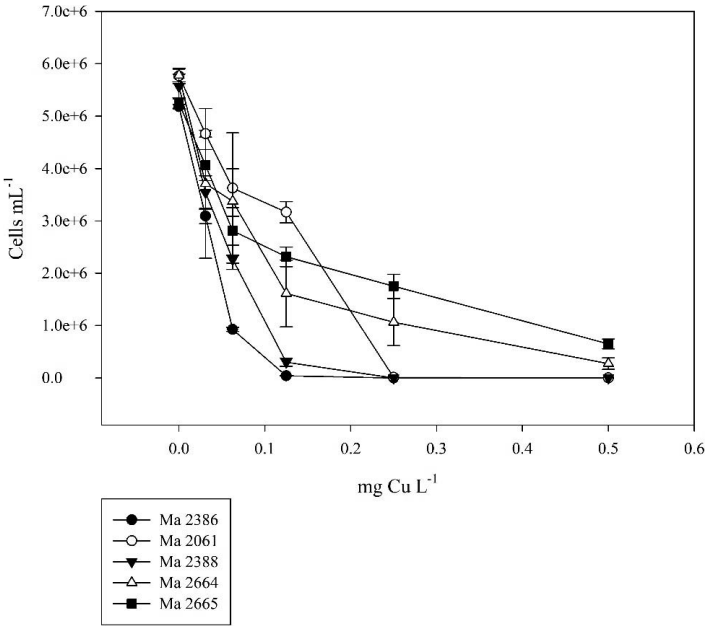

Figure 8. Mean cell densities of five *Microcystis aeruginosa* strains grown in low-P media after a 96-h exposure to copper sulfate pentahydrate. Error bars represent 1 standard error from the mean.

synthesis, respiration, enzyme function, and adenosine triphosphate production (Stauber and Florence 1987). Accumulated polyphosphate bodies have a high affinity for metals, and have been shown to sequester and ameliorate toxicity (Twiss and Nalewajko 1992, Verma et al. 1993, Chaffin et al. 2011). Naturally present copper-resistant cells have also been documented in field *M. aeruginosa* populations, but they exhibited decreased fitness in the absence of copper (Garcia-Villada et al. 2004). Decreasing management dependency on copper sulfate use can reduce the likelihood of forming a more tolerant *M. aeruginosa* population. This research revealed differences in susceptibility to copper among strains and variability in the

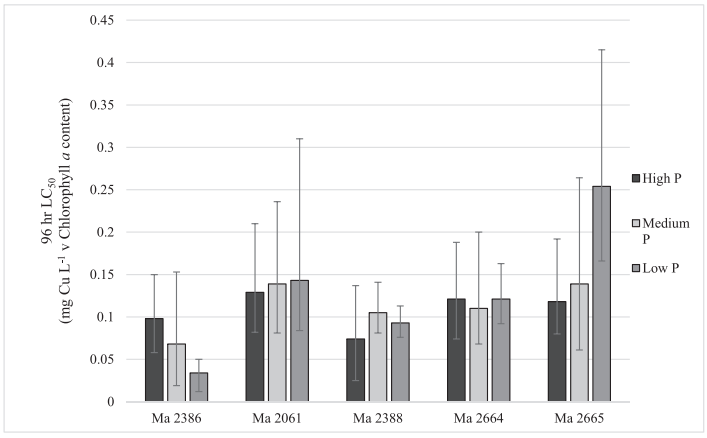

Figure 9. Cumulative mean 96-h 50% lethal concentration (LC_{50}) values based on chlorophyll *a* content for five *Microcystis aeruginosa* strains grown in high-, medium-, and low-P media after exposure to copper sulfate pentahydrate. Error bars represent 95% confidence intervals around the mean.

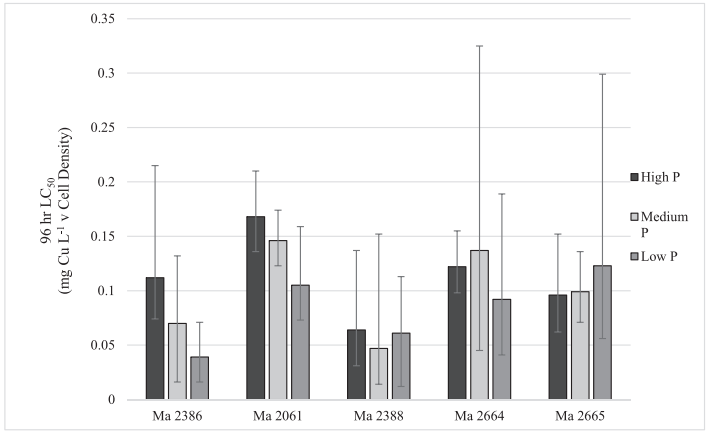

Figure 10. Cumulative mean 96-h 50% lethal concentration (LC_{50}) values based on cell densities for five *Microcystis aeruginosa* strains grown in high-, medium-, and low-P media after exposure to copper sulfate pentahydrate. Error bars represent 95% confidence intervals around the mean.

alteration of susceptibility based on accumulated P content. Copper detoxification by P compounds may allow for sustained blooms in elevated P conditions (Zeng et al. 2009), further decreasing the efficiency of copper at achieving control and increasing the continued need for reactive management. Such a situation could further propagate naturally occurring copper-resistant cells, and increase the likelihood of copper-resistant populations (Garcia-Villada et al. 2004). Incorporating strategic P mitigation may enable increased efficacy of reactive management.

Zeng et al. (2009) found much higher uptake of metals (cadmium, zinc) with increasing ambient P concentrations in culture media, and P-replete cultures were more tolerant to these metal exposures. In this research, many strains did not exhibit increased sensitivity to copper under low P, suggesting that copper may parallel P in cellular internalization. Increased copper uptake with higher-P cultures may correspond to their internal capability to detoxify copper in polyphosphate granules. In other work, cyanobacteria grown under P-replete conditions accumulated more heavy metals in polyphosphate granules than P-depleted cells (Verma et al. 1993). Cells grown in low-P conditions may have less copper uptake and less ability to ameliorate internal copper effects. Sustained, elevated growth rates in high-P cultures (> 0.1 d^{-1}, except for *M. aeruginosa* 2664) throughout the testing duration may have promoted uptake of copper, though simultaneous uptake of P may have offset toxicity. Stationary growth phase in the low-P cultures (growth rates < 0.05 d^{-1}) likely decreased copper and P uptake as well as the amount of ions internalized by the cells. Specific measurement of copper uptake in different P growth regimes will be addressed in future works. Verma et al. (1991, 1993) found less copper internalized by P-depleted cells in comparison with P-replete cells. They hypothesized that phosphate plays an important role in regulating uptake of copper in low-phosphate conditions. In this study, cellular P contents were inversely correlated with susceptibility to copper exposure for only one strain (*M. aeruginosa* 2386). Increased growth rates were measured for all *M.*

Table 3. Calculated 96-h 50% lethal concentration (LC_{50}) values for *Microcystis aeruginosa* chlorophyll *a* content and cell densities. Range represents the 95% confidence interval for the nonlinear (polynomial, quadratic) regression analysis. The corresponding R^2 value is also given.

	Chlorophyll *a* 96-h LC_{50}	95% Confidence Limit	R^2	Cell Density 96-h LC_{50}	95% Confidence Limit	R^2
Strain 2386						
High	0.098	0.058–0.150	0.8658	0.112	0.074–0.215	0.9954
Medium	0.068	0.019–0.153	0.7523	0.070	0.016–0.132	0.8638
Low	0.034	0.012–0.050	0.8694	0.039	0.016–0.071	0.8949
Strain 2061						
High	0.129	0.082–0.210	0.9403	0.168	0.136–0.210	0.9887
Medium	0.139	0.081–236	0.9067	0.146	0.123–0.174	0.9920
Low	0.143	0.084–310	0.8862	0.105	0.073–0.159	0.9517
Strain 2388						
High	0.074	0.025–0.137	0.8764	0.064	0.031–0.137	0.8155
Medium	0.105	0.081–0.141	0.9719	0.047	0.014–0.152	0.8157
Low	0.093	0.076–0.113	0.9854	0.061	0.012–0.113	0.8453
Strain 2664						
High	0.121	0.074–0.188	0.8155	0.122	0.098–0.155	0.9427
Medium	0.110	0.068–0.200	0.9048	0.137	0.045–0.325	0.9035
Low	0.121	0.092–0.163	0.9672	0.092	0.041–0.189	0.8740
Strain 2665						
High	0.118	0.080–0.192	0.9409	0.096	0.062–0.152	0.9356
Medium	0.139	0.061–0.264	0.8754	0.099	0.071–0.136	0.9642
Low	0.254	0.166–0.415	0.9365	0.123	0.056–0.299	0.8441

aeruginosa strains in increasing P concentrations, which may result in a shorter interval between reactive treatments to maintain the designated uses of the water resource (Table 1). Higher abundance (cells or biomass) can alter the mass of copper required to control the population (Bishop and Rodgers 2012). By further understanding interactions among aqueous P concentration, cellular P contents, and response of *M. aeruginosa*, the amount of copper needed for desired control in different environmental conditions can be better predicted.

Future research directions

Although both nitrogen (N) and P have been indicated in supporting *M. aeruginosa* blooms (Chaffin et al. 2013, Monchamp et al. 2014), difficulty exists in limiting *in situ* N to *M. aeruginosa*, especially since previously fixed atmospheric N can be used as a source (Beversdorf et al. 2013). Elevated N supply (Watanabe and Oishi 1985, Horst et al. 2014, Beversdorf et al. 2015) or decreased N : P ratios have increased toxin production in *M. aeruginosa* (Orihel et al. 2012, Harris et al. 2014). Microcystins have been implicated in hydroxyl free-radical abatement (Zilliges et al. 2011, Meissner et al. 2013), which can result from oxidized glutathione after copper exposures (Stauber and Florence 1987; Tripathi et al. 2006). Thus, N levels and N-rich cyanotoxins may affect copper toxicity, and will be specifically investigated in future work. *Microcystis aeruginosa* 2386 was the only strain tested that is not known to produce any microcystins, and it was the only strain that showed significantly altered sensitivity to copper depending on the P concentration. *Microcystis aeruginosa* 2061 and *M. aeruginosa* 2386 cultures had nondetect microcystins (< 0.1 µg/L) throughout the study. *Microcystis aeruginosa* 2665 cultures ranged from 12.3 to 20.1 µg microcystins L^{-1}, *M. aeruginosa* 2664 cultures ranged from 2.8 to 20.0 µg microcystins L^{-1}, and *M. aeruginosa* 2388 cultures ranged from 0.6 to 12.9 µg microcystins L^{-1}. If toxin can ameliorate oxidative stress after algaecide exposures (e.g., copper or peroxide), selection for toxic strains may result (Burkholder and Glibert 2006). Physiological changes in *M. aeruginosa* strains, as governed by site-specific environmental conditions (Jacoby et al. 2000), need to be further understood to reliably predict responses of these noxious cyanobacteria to management approaches.

In this research, we assessed exposure dynamics that incorporated innate sensitivities of different *M. aeruginosa* strains to copper sulfate, and influence of the P concentration. P-depleted cells had decreased growth rates, chlorophyll *a* content, and P content in comparison with P-replete cells, across all *M. aeruginosa* strains tested. Innate sensitivities to copper differed among *M. aeruginosa* strains, and the influence of P concentrations also differed among strains. Only one strain (*M. aeruginosa* 2386) had an increased sensitivity to copper in a low-P growth concentration. Incorporating P mitigation can allow for more strategic and efficient use of copper algaecides. By understanding the specific algal strain and P concentration, management effectiveness can be better predicted.

Since environmental conditions can alter responses of *M. aeruginosa* populations to copper application, it is important for water resource managers to consider these factors in designing an appropriate management strategy. This research can guide management recommendations to balance the ability to attain control using copper, with the benefits of P mitigation. In some situations, such as with strain *M. aeruginosa* 2386, a hybrid approach to management (e.g., P reduction and copper algaecide application) may decrease the environmental burden of copper needed to attain control. Incorporation of nutrient mitigation may also provide the benefit of long-term assemblage alteration, away from cyanobacterial dominance (Bishop and Richardson 2017). Nuisance algal blooms are dynamic in space and time, and require multidimensional management approaches to attain control, especially under changing environmental conditions (e.g., increasing temperature, carbon dioxide,

nutrients, toxins) that may diminish cyanobacterial responses to typical management approaches. This research provides insights on the direction and efficacy of an integrated approach to noxious algal management.

SOURCES OF MATERIALS

[1]Microcystins/nodularins enzyme-linked immunosorbent assay (ELISA) kit, PN 520011, Abraxis, Warminster, PA 18974.

[2]Copper sulfate pentahydrate, Fisher Scientific, Waltham, MA 02451.

[3]Trace-metal-grade nitric acid, Fisher Scientific, Waltham, MA 02451.

[4]Glass microfiber syringe 0.2 µm filter, Phenomenex, Torrance, CA 90501.

[5]Inductively coupled plasma–optical emission spectrometer, ICPE 9000, Shimadzu Corporation, Kyoto, Japan.

[6]1,000 mg Cu/L standard, Honeywell Fluka™, Morris Plains, NJ 07950.

[7]Wallac Victor^2 spectrofluorometer, Perkin Elmer, Waltham, MA 02451.

[8]Methylene blue, mortal stain, Acros Organics, Geel, Belgium.

[9]Axioskop 20, light microscope, Zeiss, Oberkochen, Germany.

[10]Improved Neubauer hemocytometer, Hausser Scientific, Horsham, PA 19044.

[11]Sulfuric acid, Fisher Scientific, Waltham, MA 02451.

[12]Konelab Aqua 420, discrete analyzer, ThermoFisher, Waltham, MA 02451.

[13]Phosphorus reference standard, Honeywell Fluka™, Morris Plains, NJ 07950.

[14]Microsoft Excel 2010, spreadsheet software, Microsoft, Redmond, WA 98052.

[15]SigmaPlot version 12.5, statistical software, Systat Software, Inc., San Jose, CA 95110.

ACKNOWLEDGEMENTS

The authors thank Clayton Lynch and Hasan Hasan for experimental and analytical support.

LITERATURE CITED

Baldia SF, Evangelista AD, Aralar EV, Santiago AE. 2007. Nitrogen and phosphorus utilization in the cyanobacterium *Microcystis aeruginosa* isolated from Laguna de Bay, Philippines. J. Appl. Phycol. 19:607–613.

Barbiero RP, Welch EB. 1992. Contribution of benthic blue-green-algal recruitment to lake populations and phosphorus translocation. Freshw. Biol. 27:249–260.

Beversdorf LJ, Miller TR, McMahon KD. 2013. The role of nitrogen fixation in cyanobacterial bloom toxicity in a temperate, eutrophic lake. PLOS ONE 8:e56103.

Beversdorf LJ, Miller TR, McMahon KD. 2015. Long-term monitoring reveals carbon–nitrogen metabolism is key to microcystin production in eutrophic lakes. Front. Ecol. 6:1–12.

Bishop WM, Richardson RJ. 2018. Influence of Phoslock on legacy phosphorus, nutrient ratios and algal assemblage composition in hypereutrophic water resources. Environ. Sci. Pollut. Res. 25:4544–4557.

Bishop WM, Rodgers JH Jr. 2012. Responses of *Lyngbya wollei* to exposures of copper-based algaecides: The critical burden concept. Arch. Environ. Contam. Toxicol. 62:403–410.

Burkholder JM, Glibert PM. 2006. Intraspecific variability: An important consideration in forming generalizations about toxigenic algal species. Afr. J. Mar. Sci.28:177–180.

Carmichael WW. 2001. Assessment of blue-green algal toxins in raw and finished drinking water. AWWA Research Foundation. American Water Works Association, Denver.

Chaffin JD, Bridgeman TB, Bade DL. 2013. Nitrogen constrains the growth of late summer cyanobacterial blooms in Lake Erie. Adv. Microbiol. 3:16–26.

Chaffin JD, Bridgeman TB, Heckathorn SA, Mishra S. 2011. Assessment of *Microcystis* growth rate potential and nutrient status across a trophic gradient in western Lake Erie. J. Great Lakes Res. 37:92–100.

Chorus I, Bartram JE. 1999. Toxic cyanobacteria in water: A guide to public health significance, monitoring and management. E&FN Spon, London.

Corradi MG, Gorbi G. 1993. Chromium toxicity on two linked trophic levels. II. Morphophysiological effects on *Scenedesmus acutus*. Ecotox. Environ. Safe. 25:72–78.

Downing JA, Watson SB, McCauley E. 2001. Predicting cyanobacteria dominance in lakes. Can. J. Fish Aquat. Sci. 58:1905–1908.

Franklin NM, Stauber JL, Apte SC, Lim RP. 2002. Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays. Environ. Toxicol. Chem. 21:742–51.

Ganf GG, Oliver RL. 1982. Vertical separation of the light and available nutrients as a factor of causing replacement of green algae by blue-green algae in the plankton of a stratified lake. J. Ecol. 70:829–844.

García-Villada L, Rico M, Altamirano M, Sánchez-Martín L, López-Rodas V, Costas E. 2004. Occurrence of copper resistant mutants in the toxic cyanobacteria *Microcystis aeruginosa*: Characterisation and future implications in the use of copper sulphate as algaecide. Water Res. 38:2207–2213.

Grantz EM, Haggard BE, Scott JT. 2013. Stoichiometric imbalance in rates of nitrogen and phosphorus retention, storage, and recycling can perpetuate nitrogen deficiency in highly productive reservoirs. Limnol. Oceanogr. 59:2203–2216.

Guasch H, Navarro E, Serra A, Sabater S. 2004. Phosphate limitation influences the sensitivity to copper in periphytic algae. Freshw. Biol. 49:463–473.

Hallegraeff GM. 1993. A review of harmful algal blooms and their apparent global increase. Phycologia 32:79–99.

Han FX, Hargreaves JA, Knigery WL, Huggett DB, Schlenk DK. 2001. Accumulation, distribution, and toxicity of copper in sediments of catfish ponds receiving periodic copper sulfate applications. J. Environ. Qual. 30:912–919.

Hansen JA, Rose JD, Jenkins RA, Gerow KG, Bergman HL. 1999. Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*) exposed to copper: Neurophysiological and histological effects on the olfactory system. Environ. Toxicol. Chem. 18(9):1979–1991.

Harke MJ, Berry DL, Ammerman JW, Gobler CH. 2012. Molecular response of the bloom-forming cyanobacterium, *Microcystis aeruginosa*, to phosphorus limitation. Microb. Ecol. 63:188–189.

Harke MJ, Gobler CJ. 2013. Global transcriptional responses of the toxic cyanobacterium, *Microcystis aeruginosa*, to nitrogen stress, phosphorus stress, and growth on organic matter. PLOS ONE 8:7.

Harris TD, Wilhem FM, Graham JL, Loftin KA. 2014. Experimental manipulation of TN:TP ratios suppress cyanobacterial biovolume and microcystin concentration in large-scale mesocosms. Lake Reserv. Manage. 30:72–83.

Hart MR, Quin BF, Nguyen ML. 2004. Phosphorus runoff from agricultural lands and direct fertilizer effects: A review. J. Environ. Qual. 33:1954–1972.

Haughey MA, Anderson MA, Whitney RD, Taylor WD, Losee RF. 2000. Forms and fate of Cu in a source drinking water reservoir following $CuSO_4$ treatment. Water Res. 34:3440–3452.

Heisler J, Glibert PM, Burkholder JM, Anderson DA, Cochlan WP, Dennison WC, Dortch Q, Gobler C, Heil CA, Humphries E. 2008. Eutrophication and harmful algal blooms: A scientific consensus. Harmful Algae 8:3–13.

Horst GP, Sarnelle O, White JD, Hamilton SK, Kaul RB, Bressie JD. 2014. Nitrogen availability increases the toxin quota of a harmful cyanobacterium, *Microcystis aeruginosa*. Water Res. 54:188–198.

Hrudey S, Burch S, Burch M, Drikas M, Greorgy R. 1999. Remedial measures. In: I.Chorus, J. Bartram, (*eds.*). Toxic cyanobacteria in water. A guide to their public health consequences, monitoring and management. Routledge, London.

Hutchinson TC, Stokes PM. 1975. Heavy metal toxicity and algal bioassays. American Society for Testing and Materials; special technical publication 573:320–343.

Jacob AP, Culver DA, Lanno RP, Voigt A. 2016. Ecological impacts of fluridone and copper sulphate in catfish aquaculture ponds. Environ. Toxicol. Chem. 35:1183–1194.

Jacobson L, Halmann M. 1982. Polyphosphate metabolism in the blue-green alga *Microcystis aeruginosa*. J. Plankton Res. 4:481–488.

Jacoby CA, Frazer TK. 2009. Eutrophication: Time to adjust expectations. Science 324:723–724.

Jacoby JM, Collier DC, Welch EB, Hardy FJ, Crayton M. 2000. Environmental factors associated with a toxic bloom of *Microcystis aeruginosa*. Can. Spec. Publ. Fish Aquat. Sci. 57:231–240.

Jensen TE, Baxter M, Rachlin JW, Jani V. 1982. Uptake of heavy metals by *Plectonema boryanum* (Cyanophycea) into cellular components, especially polyphosphate bodies: An X-ray energy dispersive study. Environ. Pollut. A Ecol. Biol. 27:119–127.

Ji Y, Sherrell RM. 2008. Differential effects of phosphorus limitation on cellular metals in *Chlorella* and *Microcystis*. Limnol. Oceanogr. 53:1790–1804.

Kilham SS, Kreeger DA, Lynn SG, Goulden CE, Herrera L. 1998. COMBO: A defined freshwater culture medium for algae and zooplankton. Hydrobiologia 377:147–159.

Kromkamp J, Van Den Heuvel A, Mur LR. 1989. Phosphorus uptake and photosynthesis by phosphate-limited cultures of the cyanobacterium *Microcystis aeruginosa*. Br. Phycol. J. 24:347–355.

Lakeman MB, von Dassow P, Cattolico RA. 2009. The strain concept in phytoplankton ecology. Harmful Algae 8:746–758.

Lampert W. 1982. Further studies on the inhibitory effect of the toxic blue-green *Microcystis aeruginosa* on the filtering rate of zooplankton. Arch. Hydrobiol. 95:207–220.

Lewis PA, Klemm DJ, Lazorchak JM, Norberg-King TJ, Peltier TJ, Peltier WH, Heber MA. 1994. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms (Green alga, *Selenastrum capricornutum*, Growth 1003.0). 3rd ed. U.S. Environmental Protection Agency, Washington, DC. EPA/600.4-91/002.

Liu W, Qiao Q, Chen Y, Wu K, Zhang X. 2014. Microcystin-LR exposure to adult zebrafish (*Danio rerio*) leads to growth inhibition and immune dysfunction in F1 offspring, a parental transmission effect of toxicity. Aquat. Toxicol. 155:360–367.

Lombardi AT, Wangersky P. 1991. Influence of phosphorus and silicon on lipid class production by the marine diatom *Chaetoceros gracilis* grown in turbidostat cage cultures. Mar. Ecol. Prog. Ser. 77:39–47.

Mastin BJ, Rodgers JH Jr. 2000. Toxicity and bioavailability of copper herbicides (Clearigate, Cutrine Plus, and copper sulfate) to freshwater animals. Arch. Environ. Contam. Toxicol. 39:445–451.

Mastin BJ, Rodgers JH, Jr., Deardorff TL. 2002. Risk evaluation of cyanobacteria-dominated algal blooms in a North Louisiana reservoir. J. Aquat. Ecosyst. Stress Recov. 9:103–114.

McDonald, JH. 2014. Handbook of biological statistics. 3rd ed. Sparky House Publishing, Baltimore.

Meissner S, Fastner J, Dittmann E. 2013. Microcystin production revisited: Conjugate formation makes a major contribution. Environ. Microbiol. 15:1810–1820.

Monchamp M-E, Pick FR, Beisner BE, Maranger R. 2014. Nitrogen forms influence microcystin concentration and composition via changes in cyanobacterial community structure. PLOS ONE 9: e85573.

Nagai T, Imai A, Matsushige K, Fukushima T. 2007. Growth characteristics and growth modeling of *Microcystis aeruginosa* and *Planktothrix agardhii* under iron limitation. Limnology 8:261–270.

Oliveira-Filho EC, Lopes RM, Paumgartten FJR. 2004. Comparative study on the susceptibility of freshwater species to copper-based pesticides. Chemosphere 56:369–374.

Orihel DM, Bird DF, Brylinsky M, Chen H, Donald DB, Huang DY, Giani A, Kinniburgh D, Kling H, Kotak BG, Leavitt PR. 2012. High microcystin concentrations occur only at low nitrogen-to-phosphorus ratios in nutrient-rich Canadian lakes. Can. J. Fish. Aquat. Sci. 69:1457–1462.

Paerl HW. 1988. Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. Limnol. Oceanogr. 33:823–847.

Reynolds CS. 2006. Ecology of phytoplankton. Cambridge Univ. Press, New York. 552 pp.

Reynolds CS, Walsby AE. 1975. Water blooms. Biol. Res. 50:437e481.

Rhee G-Y. 1972. Competition between an alga and an aquatic bacterium for phosphate. Limnol. Oceanogr. 7:505–514.

Rhee G-Y. 1973. A continuous culture study of phosphate uptake, growth rate and polyphosphate in *Scenedesmus* sp. J. Phycol. 9:495–506.

Rhee G-Y. 1982. Effects of environmental factors and their interactions on phytoplankton growth. In: K. C. Marshall (ed.). Advances in microbial ecology. Plenum Press, New York. pp. 33–74.

Rhee G-Y, Gotham IJ. 1981. The effect of environmental factors on phytoplankton growth: temperature and the interactions of temperature with nutrient limitation. Limnol. Oceanogr. 26:635–648.

Rocha GS, Lombardi AT, Melão Mda G. 2016. Influence of phosphorus on copper toxicity to *Selenastrum gracile* (Reinsch) Korshikov. Ecotox. Environ. Safe. 128:30–35.

Saxton MA, Arnold RJ, Bourbonniere RA, McKay RML, Wilhelm SW. 2012. Plasticity of total and intracellular phosphorus quotas in *Microcystis aeruginosa* cultures and Lake Erie algal assemblages. Front. Microbiol. 3:1–7.

Saxton MA, Morrow EA, Bourbonniere RA, Wilhelm SW. 2011. Glyphosate influence on phytoplankton community structure in Lake Erie. J. Great Lakes Res. 37:683–690.

Seale DB, Boraas ME, Warren GJ. 1987. Effects of sodium and phosphate on growth of cyanobacteria. Water Res. 21:625–631.

Serra A, Guasch H, Admiraal W, Van der Geest HG, Van Beusekom SAM. 2010. Influence of phosphorus on copper sensitivity of fluvial periphyton: The role of chemical, physiological and community-related factors. Ecotoxicology 19:770–780.

Shen H, Song L. 2007. Comparative studies on physiological responses to phosphorus in two phenotypes of bloom-forming *Microcystis*. Hydrobiologia 592:475–486.

[SMEWW] Standard Methods for the Examination of Water and Wastewater. 2005. Standard Methods for the Examination of Water and Wastewater. 21st ed. American Public Health Association, Washington, DC.

Smith VH. 1983. Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. Science 221:669–671.

Søndergaard M, Jensen PJ, Jeppesen E. 2001. Retention and internal loading of phosphorus in shallow, eutrophic lakes. Sci. World J. 1:427–442.

Stauber JL, Florence TM. 1987. Mechanism of toxicity of ionic copper and copper complexes to algae. Mar. Biol. 94:511–519.

Sterner RW. 2008. On the phosphorus limitation paradigm for lakes. Int. Rev. Hydrobiol. 93:433–445.

Stoddard JL, Van Sickle J, Herlihy AT, Brahney J, Paulsen S, Peck DV, Mitchell R, Pollard AI. 2016. Continental-scale increase in lake and stream phosphorus: Are oligotrophic systems disappearing in the United States? Environ. Sci. Technol. 50(7):3409–3415.

Štrojsová A, Vrba J, Nedoma J, Šimek K. 2005. Extracellular phosphatase activity of freshwater phytoplankton exposed to different *in situ* phosphorus concentrations. Mar. Freshw. Res. 56:417–424.

Tilman D, Kiesling R, Sterner R, Kilham SS, Johnson FA. 1986. Green, blue-green and diatom algae: Taxonomic differences in competitive ability for phosphorus, silicon and nitrogen. Archiv. Hydrobiol. 106:473–485.

Tilman D, Kilham SS, Kilham P. 1982. Phytoplankton community ecology: The role of limiting nutrients. Annu. Rev. Ecol. Syst. 13:349–372.

Tripathi BN, Mehta SK, Amar A, Gaur JP. 2006. Oxidative stress in *Scenedesmus* sp. during short- and long-term exposure to Cu^{+2} and Zn^{+2}. Chemosphere 62:538–544.

Twiss MR, Nalewajko C. 1992. Influence of phosphorus nutrition on copper toxicity to three strains of *Scenedesmus acutus* (Chlorophyceae). J. Phycol. 28:291–298.

Twiss MR, Welbourn PM, Schwartzel E. 1993. Laboratory selection for copper tolerance in *Scenedesmus acutus* (Chlorophyceae). Can. J. Bot. 71:333–338.

[USEPA] U.S. Environmental Protection Agency. 1978. Method 365.3 Phosphorous, All Forms (Colorimetric, Ascorbic Acid, Two Reagent). Washington, DC.

[USEPA] U.S. Environmental Protection Agency. 2007. National Lakes Assessment: Field Operations Manual. EPA 841-B-07-004 U.S. Environmental Protection Agency, Office of Water and Office of Research and Development. Washington, DC.

[USEPA] U.S. Environmental Protection Agency. 2011. National Pollutant Discharge Elimination System (NPDES). Pesticide General Permit (PGP) for Discharges from the Application of Pesticides. Washington, DC.

[USEPA] U.S. Environmental Protection Agency. 2016. National Lakes Assessment 2012: A Collaborative Survey of Lakes in the United States. EPA 841-R-16-113. U.S. Environmental Protection Agency, Washington, DC. https://nationallakesassessment.epa.gov/. Accessed October 15, 2017.

Van Mooy BAS, Fredricks HL, Pedler BE, Dyhrman ST, Karl DM, Koblížek M, Lomas, MW, Mincer TJ, Moore LR, Moutin T, Rappé MS, Webb EA. 2009. Phytoplankton in the ocean use non-phosphorus lipids in response to phosphorus scarcity. Nature 458:69–72.

van Oosterhout F, Lürling M. 2013. Phosphorus binding clay (Phoslock) in mitigating cyanobacterial nuisance: Effects on water quality variables and plankton. Hydrobiologia 710:265–277.

Verma SK, Singh RK, Singh SP. 1993. Copper toxicity and phosphate utilization in the cyanobacterium *Nostoc calcicola*. Bull. Environ. Contamin. Toxicol. 50:192–198.

Verma SK, Singh SP, Singh RK. 1991. Nutritional control of copper uptake in the cyanobacterium *Nostoc calcicola* Breb. BioMetals 4:192–196.

Wang W-X, Dei RCH. 2006. Metal stoichiometry in predicting Cd and Cu toxicity to a freshwater green alga *Chlamydomonas reinhardtii*. Environ. Poll. 142:303–312.

Watanabe MF, Oishi S. 1985. Effects of environmental factors on toxicity of a cyanobacterium (*Microcystis aeruginosa*) under culture conditions. Appl. Environ. Microbiol. 49:1342–1344.

Watson SB, McCauley E, Downing JA. 1997. Patterns in phytoplankton taxonomic composition across temperate lakes of differing nutrient status. Limnol. Oceanogr. 42:487–495.

Whitton BA, Grainger SLJ, Hawley GRW, Simon JW. 1991. Cell-bound and extracellular phosphatase activities of cyanobacterial isolates. Microb. Ecol. 21:85–98.

Wu H, Wei G, Tan X, Li L, Li M. 2017. Species-dependent variation in sensitivity of *Microcystis* species to copper sulfate: Implication in algal toxicity of copper and controls of blooms. Sci. Rep. 7:40393.

Wu ZX, Gan NQ, Huang Q, Song L-R. 2007. Response of *Microcystis* to copper stress—Do phenotypes of *Microcystis* make a difference in stress tolerance? Environ. Pollut. 147:324–330.

Yang YY, Lusk MG. 2018. Nutrients in urban stormwater runoff: Current state of the science and potential mitigation options. Curr. Pollut. Rep. 4:112–127.

Zeng J, Yang L, Wang WX. 2009. Cadmium and zinc uptake and toxicity in two strains of *Microcystis aeruginosa* predicted by metal free ion activity and intracellular concentration. Aquat. Toxicol. 91:212–220.

Zhao SJ, Xie P, Li G, Chen J, Cai Y, Xiong Q, Zhao YY. 2012. The proteomic study on cellular responses of the testes of zebrafish (*Danio rerio*) exposed to microcystin-RR. Proteomics 12:300–312.

Zilliges Y, Kehr J-C, Meissner S, Ishida K, Mikkat S, Hagemann M, Kaplan A, Boerner T, Dittmann E. 2011. The cyanobacterial hepatotoxin microcystin binds to proteins and increases the fitness of *Microcystis* under oxidative stress conditions. PLOS ONE 6:e17615.