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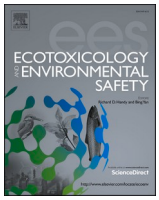

Efficacy of algaecides for the proactive treatment of overwintering cyanobacteria

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ABSTRACT

Once established within a water resource, harmful algal blooms (HABs) can occur seasonally with an intense and rapid onset, giving water resource managers limited time to respond to lessen risks. An attractive strategy to decrease human, ecological, and economic risks from HABs is to implement proactive algaecide treatments applied to overwintering cyanobacteria (i.e., akinetes and quiescent vegetative cells) in sediments prior to the formation of a HAB; however, this approach is novel and very limited efficacy data exist. Therefore, the specific objectives of this research were to 1) evaluate copper- and peroxide-based algaecides, applied as single and repeat treatments at the bench scale, to identify effective proactive treatments, and 2) compare correlations between cell density and other response measurements (i.e., in vivo chlorophyll *a* and phycocyanin concentrations and percent benthic coverage), to identify informative metrics to assess overwintering cyanobacteria responses. Twelve treatment scenarios using copper- and peroxide-based algaecides were applied to sediments containing overwintering cyanobacteria prior to a 14 d incubation under favorable growth conditions. Responses of cyanobacteria in the planktonic (i.e., cell density, in vivo chlorophyll *a* and phycocyanin concentrations) and benthic (percent coverage) phases after a 14 d incubation were evaluated in treatments and controls. The HAB-forming cyanobacteria present after a 14 d incubation were: *Aphanizomenon, Dolichospermum*, *Microcystis, Nostoc*, and *Planktonthrix*. Successive treatments of copper sulfate (CuSulfate) followed by sodium carbonate peroxyhydrate (PeroxiSolid) (second algaecide applied after 24 h) as well as repeat applications of a single algaecide, PeroxiSolid (second treatment applied after 24 h) resulted in statistically significant ($p \leq 0.05$; $\alpha = 0.05$) declines in cell density relative to untreated controls. Planktonic cyanobacteria responses measured in terms of phycocyanin concentrations were strongly correlated with cyanobacteria density measurements (Pearson's correlation coefficient (r) = 0.89). Chlorophyll *a* concentrations and percent benthic coverage did not correlate with planktonic cyanobacteria density measurements (r = 0.37 and −0.49, respectively) and therefore, were unreliable metrics for cyanobacterial responses in this study. These data provide initial evidence of the efficacy of algaecides for treating overwintering cells in sediments and contribute to our overarching hypothesis that proactive treatments may delay the onset and intensity of HABs in impacted waterbodies.

1. Introduction

Inland freshwaters are a critical economic, ecological, and public health resource; yet they can experience intense and rapid onsets of harmful algal blooms (HABs) which impair these services (Boyer et al., 2008; Coffer et al., 2020; Graham et al., 2017; Walker et al., 2008). An explanation for this phenomenon is that once harmful algae (i.e., cyanobacteria) become established within a water resource, cells remain quiescent in sediments during non-ideal growth conditions and proliferate during the growing season, providing an inoculum for rapid HAB formation. These quiescent cells are termed overwintering cells in this study and include both specialized cells or akinetes of the order

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Nostocales (e.g., *Aphanizomenon* and *Dolichospermum*) and vegetative cyanobacteria cells that remain associated with the sediments during unfavorable growth conditions (e.g., *Microcystis*). Recruitment of overwintering cells from the sediment to surface waters has repeatedly been identified as a contributing factor for blooms in warmer seasons (Kaplan-Levy et al., 2010; Kim et al., 2005; Kitchens et al., 2018). Yet, information is lacking to inform management of overwintering cells to mitigate recruitment and subsequently lessen risks (e.g., human and ecological health, economic) posed by HABs (Calomeni et al., 2022). Therefore, there is a need to investigate novel treatment strategies targeting the proactive management of HABs at early stages of cyanobacterial growth.

Treatment strategies can generally be described as chemical, mechanical, or biological (ITRC 2021); however, to investigate the efficacy of this proactive strategy, this study focused on chemical control or copper- and peroxide-based algaecides registered by the United States Environmental Protection Agency (USEPA) (one algaecide currently in USEPA review). Algaecides are most commonly used when cyanobacteria achieve visually dense growths in the planktonic phase and are often effective mitigation tools to diminish cell densities rapidly (over hours or days). However, a limitation of algaecides in some cases is the durability of treatment, in terms of sustaining longer term efficacy. Therefore, a potentially overlooked opportunity is to use algaecides prior to the germination and growth of overwintering cells to treat these cells and decrease recruitment to the planktonic phase. If successful, this approach may provide an improved treatment strategy by avoiding or delaying HAB impairment while concomitantly being more cost-effective.

Both the physiology of overwintering cells and their location in sediments may present challenges to maintain sufficient algaecide activity for effective proactive treatments (Calomeni et al., 2022). For the main two algaecide active ingredients registered in the US (copper- and peroxide-based algaecides), sediments may decrease cyanobacteriacidal activity. For copper-based algaecides, sediments may provide a source of ligands to sorb the active ingredient (i.e., copper) (Calomeni et al., 2017; Willis and Bishop, 2016). Because peroxide-based algaecides are oxidants, organic matter in sediments may also be oxidized and decrease activity of the active ingredient (Finnegan et al., 2010) prior to oxidation of target cells. Another challenge to efficacy that must be overcome is that both algaecides are anticipated to elicit detrimental effects when sufficient active ingredient interacts with reactions (e.g., photosynthesis and respiration) and compounds (e.g., amino acids, proteins) internal to the cell (Drábková et al., 2007; Finnegan et al., 2010; Russell et al., 2003). However, akinetes have a protective envelope (Fay, 1988) that may decrease internalization of the active ingredient and consequently efficacy. Because of these anticipated challenges, different algaecide products and repeat treatments were evaluated in the current study, at a range of concentrations allowable per algaecide labels (i.e., environmentally relevant concentrations).

Protocols for bench-scale algaecide efficacy testing are well established in the literature for planktonic and benthic cyanobacteria (Bishop and Rodgers, 2011; Calomeni et al., 2015; Calomeni et al., 2018; Geer et al., 2017; Kinley-Baird et al., 2021). Results from these types of evaluations can be used to make field predictions regarding algaecide effectiveness in situ and have been useful in the identification of effective algaecide treatments for benthic cyanobacteria that are particularly challenging to manage (e.g., *Microseira wollei* [formerly *Lyngbya wollei*]), as they allow for different exposures to be evaluated in a replicated setting (Calomeni et al., 2015; Duke, 2007). This tiered strategy provides increased confidence in field efficacy (i.e., decreases uncertainties), informs algaecide application rates and timing, and can inform margins of safety for non-target species (Calomeni et al., 2015).

Reliable and informative cyanobacteria responses are needed to discern algaecide efficacy. Of interest for this research are the number of overwintering cells that can germinate, divide, and transfer to the water column, potentially contributing to a planktonic HAB. Yet, by only measuring post-treatment overwintering cell density in sediments, the treatment efficacy could be overestimated. Specifically, overwintering cells in sediments may be counted as part of the cell density measurement that are incapable of germinating and transferring to the planktonic phase. This may be especially the case following proactive algaecide treatments targeting the sediment in which overwintering cells are made non-viable. Therefore, measurements of overwintering cell responses to algaecides should assess recruitment and growth from sediments. Measurements of the planktonic cyanobacteria that were capable of recruitment following an incubation period under realistic and favorable growth conditions can be used to assess the cyanobacteria of interest for this research.

A variety of measures have been used to assess responses of planktonic cyanobacteria and algae to algaecide exposures (Calomeni et al., 2014; Calomeni et al., 2018). Cell density measurements use light microscopy to identify algae on a cell-by-cell basis and can be used to discern responses of potentially harmful cyanobacteria from other algae (e.g., diatoms, green algae) in natural samples. Cell density is informative as a response measurement for this research and for comparison with other response measurements. However, cell density measurements require observation by a technician trained in algal identification, and there is interest in more rapid response measurements. Measurements of photosynthetic pigments contained by algae (i.e., chlorophyll *a*) or cyanobacteria (i.e., phycocyanin) are commonly used. These aggregate measurements of algae or cyanobacteria can be rapidly analyzed in vivo using a fluorescence-based sensor (Gregor and Maršálek, 2004). Additionally, since overwintering cells are associated with the sediment, response measurements in the benthic phase (i.e., percent benthic coverage) (Calomeni et al., 2022) may be positively related to responses in the planktonic phase. Correlations with cell density were used to compare response measurements to identify informative metrics for assessing cyanobacteria that germinated and grew from overwintering cells.

The overall goal of this research was to conduct bench-scale experiments to evaluate whether copper-and peroxide-based algaecides can be used to proactively treat overwintering cells in sediment at concentrations allowable per algaecide labels. The specific objectives of this research were to 1) evaluate single and repeat treatments of copper- and peroxide-based algaecides for their efficacy against overwintering cyanobacteria located in sediment from a HAB impacted pond and 2) compare correlations between cell density and response measurements in the planktonic (i.e., in vivo chlorophyll *a* and phycocyanin concentrations) and benthic phases (percent benthic coverage) following algaecide exposures and a 14 d incubation period.

2. Materials and methods

2.1. Study Site

Overwintering cyanobacteria and associated sediment were collected in April 2022 from the 43.3 ha Milford Lake Gathering Pond located in Junction City, KS (Fig. 1). In 2019, KDHE HAB Response Program reported microcystin concentrations in the pond of over 400 μg/L, 50 times greater than the USEPA recommended recreational ambient water quality criterion of 8 μg/L (Kansas Department of Health and Environment KDHE, 2019; USEPA, 2019; KDHE, 2022a). Severe seasonal HABs within Milford Gathering Pond have required beach closures in the summer. Potential toxin producing cyanobacteria that have been identified during seasonal monitoring are *Aphanizomenon, Dolichospermum* (formerly *Anabaena*), *Microcystis, Planktothrix,* and *Raphidiopsis* (formerly *Cylindrospermopsis*). These cyanobacteria genera are common across the conterminous United States (Beaver et al., 2018). *Aphanizomenon, Dolichospermum,* and *Raphidiopsis* are of the order Nostocales and can produce akinetes. Overwintering populations of *Microcystis* and *Planktothrix* that "seed" HABs have also been reported (Fallon and Brock, 1981; Kitchens et al., 2018; Micheletti et al., 1998;

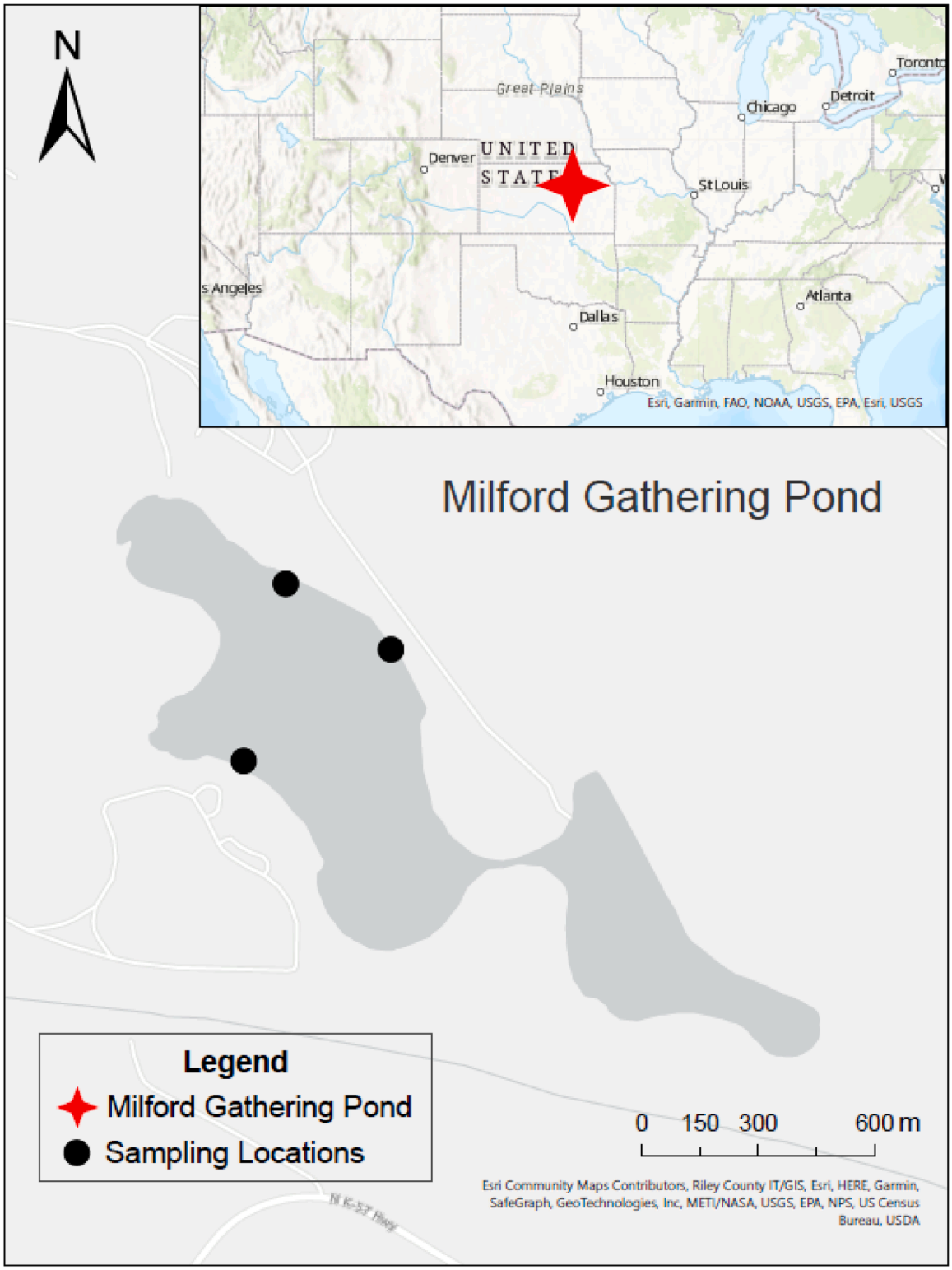

Fig. 1. Map of Milford Lake Gathering Pond, Junction City, KS, USA.

Preston et al., 1980). Akinetes and other overwintering cyanobacteria in sediments were monitored during 4 sampling events from September 2021 through March 2022 in Milford Gathering Pond and ranged from 58,744 to 327,078 cells/ g wet sediment. For context, reported overwintering cell densities in the published literature have ranged from < 150 akinetes/g sediment and < 8400 *Microcystis* cells/g sediment to 36, 000,000 akinetes/g sediment and 44,830,000 *Microcystis* cells/g sediment (Ramm et al., 2012; Cirés et al., 2013; Legrand et al., 2017).

2.2. Sediment, water andcyanobacteria sampling

Sediment samples were collected from three locations within the pond (Fig. 1), with a petite Ponar Dredge. Surficial sediments (0–2 cm from the sediment-water interface) were carefully scraped from the

sample while sediments were contained in the dredge using a plastic spatula and retained for analysis. Samples from the sediment water interface were collected because previous studies have identified that the highest densities of overwintering cells are located within this layer, and surficial cells are anticipated to be exposed to algaecides during treatment. Surficial sediment subsamples from the three locations were homogenized. Approximately 20 L of water was collected and all samples were placed on ice in a cooler for shipment overnight to the U.S. Army Engineer Research and Development Center (ERDC). Once sediments arrived at ERDC, they were stored at 4 ℃ in darkness until analysis.

2.3. Bench scale algaecide treatments

Prior to initiation of bench-scale exposures, water and sediment characteristics were measured (Table 1). Site water was vacuum filtered through a 0.45 μm pore size nitrocellulose filter paper to remove algae that would interfere with measurements of overwintering cell responses to algaecide exposures. To confirm that cyanobacteria were removed, filtered site water was placed in a Palmer-Maloney counting chamber and the entire chamber (0.1 mL) was observed. Cyanobacteria cell densities were below the detection limit (10 cells/mL). This filtered site water was used as the overlying water for the incubation study to maintain similar nutrient concentrations and ionic balance that the cells would experience in situ.

For treatments (n = 3 per treatment) and controls (n = 3 per control), ten grams of wet sediment containing 218,000 cells/ g wet sediment (± 1 SD = 3464 cells/ g wet sediment) were weighed and placed into 250 mL borosilicate glass beakers with 150 mL of filtered site water. Sediments within beakers had a depth of 0.25–0.5 cm and a surface area of 37 cm^2. To ensure that sediments were evenly distributed on the bottom of the beakers, sediments were gently stirred if necessary. Additionally, filtered site water controls (n = 3) were prepared in 250 mL beakers with chemically sterilized sand and were placed in exposure and incubation chambers together with treatments for the duration of the experiment. For the sand controls, quartz sand was sterilized using 30% hydrogen peroxide. The sand was covered with peroxide solution, stirred to suspend sand particles, and allowed to rest for 24 h. After 24 h, the peroxide solution was decanted, and the sand was thoroughly washed with deionized water.

Because of anticipated low sensitivities of overwintering cells to algaecide exposures, a suite of copper- and peroxide-based algaecides was evaluated spanning formulations available in the United States (Table 2). Algaecide formulations, including active and inactive ingredients, alter the product efficacy (Bishop et al., 2014; Calomeni et al., 2014; Calomeni et al., 2015; Kinley-Baird et al., 2021). Copper-based algaecides evaluated included a copper salt (CuSulfate) and chelated copper formulations with (CuAdjuvant) and without an adjuvant (CuChelated). Peroxide-based algaecides evaluated included solid sodium carbonate peroxyhydrate (PeroxiSolid) and two liquid formulations with 5% (PeroxiLiquid 5%PAA) and 18% peroxyacetic acid (PAA) (PeroxiLiquid 18%PAA; Table 2).

Algaecide exposures were conducted by amending site water with appropriate volumes of stock algaecide solutions (Fig. 2). Stocks consisted of 1000 mg Cu/L or 10,000 mg H_2O_2/L solutions made by weighing algaecide and adding product to 100 mL volumetric flasks containing deionized water. The solid algaecide (PeroxiSolid) was dissolved in deionized water to create the 10,000 mg H_2O_2/L stock solution. Stocks were inverted to mix thoroughly and immediately used for exposures. After amendment of the stock solution to the overlying water, the overlying water was gently stirred to homogenize without suspending sediment. Subsamples were immediately collected for confirmation of exposures (Table 2). Peroxide concentrations were measured using a thiosulfate drop count titration (Hach® Loveland, CO, USA). Copper concentrations were measured using method 6020B (USEPA, 2014).

Algaecide exposures were conducted under conditions (i.e., light and temperature) designed to simulate springtime environmental conditions within Milford Lake Gathering Pond, KS. During the April sampling event when sediments and overlying water were collected for this experiment, surface water temperatures ranged from 12.8 °C to 13.1 °C. Therefore, a reach-in Darwin Chamber® (St. Louis, MO KB034 Environmental Chamber) was maintained at 13 °C for exposures. Full-spectrum light was provided continuously by white (4000 K) LED bulbs for the duration of the study. The target nominal light intensity at the mouth of the beakers was 100 LUX.

Twenty-four hours after exposure initiation, beakers containing overwintering cells designated for repeat treatments were exposed a second time using the methods previously described (Fig. 2). Repeat treatments were used to discern potential added efficacy from treatments of two different algaecides applied 24 h apart, or from treatments of the same algaecide applied twice (24 h apart). Twenty-four hours following the final exposure, beakers were transferred to incubation chambers to begin the 14 d incubation under ideal growth conditions (Calomeni et al., 2022). Beakers were covered with a clear polyethylene film and placed in a Darwin Chamber at 25.6 °C (standard deviation = 0.1 °C). Average light intensity measured throughout the incubation period was 2617 LUX (± 589 LUX) and 1901 LUX (± 507 LUX) at the bottom of an empty 250 mL beaker and a beaker filled with 150 mL water, respectively. Beakers were maintained under consistent environmental conditions for 14 d. Fourteen days is a sufficient duration to be able to discern growth if growth was to occur based on initial assessments. After 14 d, beakers were removed from the experimental chambers, and growth responses were evaluated in the planktonic and benthic phases (Fig. 2).

Table 1
Water and sediment characteristics measured at experiment initiation and after the 14 d incubation period.

		Experiment Initiation				14 Day Incubation			
Water characteristics	**Instrument/Method**	**Minimum**	**Maximum**	**Average**	**n**	**Minimum**	**Maximum**	**Average**	**n**
pH (SU)	METTLER TOLEDO SevenCompact pH meter	7.8	9.7	7.9	48	8.5	10.3	9.3	54
Conductivity (μS/cm)	EXTECH® Instruments ExStick® II	388	461	457	48	344	650	473	54
Alkalinity (mg/L as $CaCO_3$)	HACH® Sulfuric acid drop count titration	76	236	170	45	-	-	-	-
Hardness (mg/L as $CaCO_3$)	HACH® EDTA drop count titration	68	184	125	18	-	-	-	-
Turbidity (NTU)	HACH® 2100Q Portable Turbidimeter	10.1	13.8	11.4	3	0.5	139.0	19.0	54
DO[a] (mg/L)	YSI ProODO Digital Professional Series	5.4	11.2	6.7	48	6.6	15.2	10.4	54
Sediment characteristics		**Minimum**	**Maximum**	**Average**	**n**	**Minimum**	**Maximum**	**Average**	**n**
% Solids	ASTM D2216[b]	74.3	75.2	74.8	3	-	-	-	-
% TOC[a]	Loss on ignition[c]	0.77	0.99	0.87	3	-	-	-	-

[a] Nephelometric turbidity units = NTU; Dissolved oxygen = DO; Total organic carbon = TOC
[b] ASTM 2019
[c] Schulte 1995

Table 2
Algaecide characteristics and measured exposures for bench-scale treatments.

Algaecide Type	Copper			Hydrogen Peroxide		
Product	AB™ Brand Copper Sulfate Crystals	Cutrine-Plus®	Cutrine®-Ultra	GreenClean® Pro	GreenClean Liquid 5.0	GreenClean 18[a]
Characteristics						
Formulation	Copper Sulfate Pentahydrate	Mixed Copper Ethanolamine Complex	Mixed Copper Ethanolamine Complex and D-limonene	Sodium Carbonate Peroxyhydrate	Hydrogen peroxide and Peroxyacetic acid	Hydrogen peroxide and Peroxyacetic acid
Abbreviation	CuSulfate	CuChelated	CuAdjuvant	PeroxiSolid	PeroxiLiquid 5.3%PAA	PeroxiLiquid 18%PAA
Maximum label concentration	1 mg Cu/L	1 mg Cu/L	1 mg Cu/L	10.2 mg H_2O_2/L	22.3 mg H_2O_2/L	27.5 mg H_2O_2/L
Maximum label rate	10.88 lbs of product/acre-ft	3 gallons of product/acre-ft	3 gallons of product/acre-ft	100 pounds of product/acre-ft	28.5 gallons of product/acre-ft	28.5 gallons of product/acre-ft
Average measured exposure (standard deviation)[b,c]						
Single treatment						
	0.74 (0.23) mg Cu/L	0.86 (0.01) mg Cu/L	0.83 (0.04) mg Cu/L	7.5 (1.2) mg H_2O_2/L	23.8 (2.3) mg H_2O_2/L	29.5 (1.5) mg H_2O_2/L
Repeat treatment						
First exposure: Copper or H_2O_2	0.77 (0.11) mg Cu/L	0.87 (0.03) mg Cu/L	0.83 (0.03) mg Cu/L	7.7 (1.7) mg H_2O_2/L	23.1 (3.1) mg H_2O_2/L	26.9 (1.5) mg H_2O_2/L
Second exposure:[d] H_2O_2	10.2 (0.5) mg H_2O_2/L	11.5 (1.7) mg H_2O_2/L	9.9 (0.5) mg H_2O_2/L	12.5 (2.0) mg H_2O_2/L	27.9 (1.2) mg H_2O_2/L	38.1 (3.0) mg H_2O_2/L

[a] Liquid peroxide algaecide (GreenClean 18) in review by USEPA.
[b] All exposures were applied at the maximum label concentration.
[c] Follow directions on algaecide labels for treatments within aquatic systems.
[d] The second exposure was applied 24 h after the first. For the copper-based algaecides, the second exposure was GreenClean® Pro.

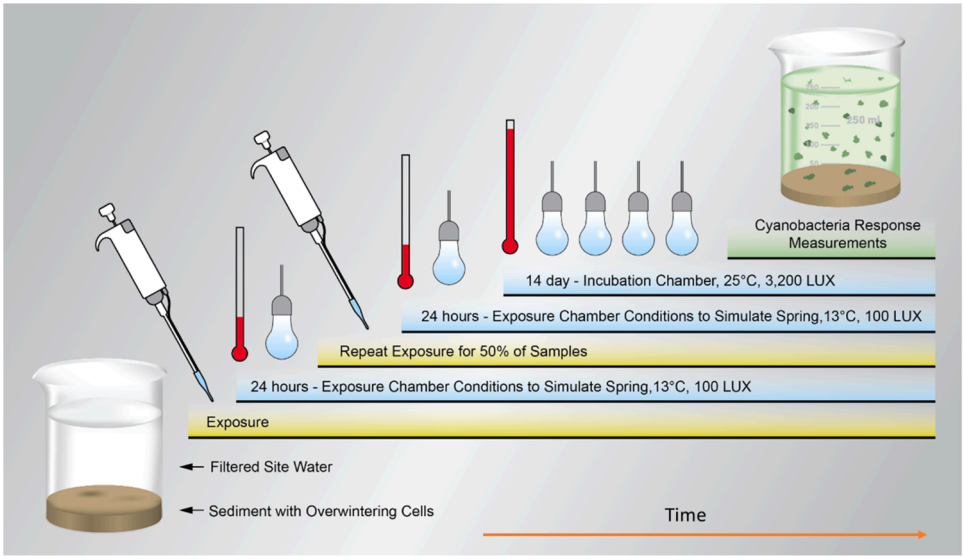

Fig. 2. Schematic presentation of the algaecide exposure and incubation experiment for bench-scale treatments.

2.4. Measurement of cyanobacteria growth responses

Responses were measured after 14 d of incubation by cyanobacterial identification and enumeration and photosynthetic pigment analysis (i.e., chlorophyll *a* and phycocyanin) in the planktonic phase. Prior to measurement of responses, the overlying water was gently stirred. This allowed for the suspension of any cyanobacteria that had settled to the sediment phase and would be resuspended with wave action in the pond. Subsamples were collected and transferred to a Palmer-Maloney counting chamber for enumeration and identification to genus. Dimensions including filament length (e.g., *Aphanizomenon*) or colony surface area (e.g., *Microcystis*) were measured using a Gryphax Arktur microscope camera (Jenoptik Huntsville, Alabama) and firmware mounted to a Motic Panthera C2 (San Antonio, TX, USA) compound microscope at 400x magnification. These dimensions were converted to a cell density by counting the number of cells per length (i.e., filaments) or surface area (i.e., colonies) of representative examples of the genera. Forty fields of view for low density samples (detection limit = 550 cells/mL) or a minimum of 40 natural units (Cottingham et al., 1998) for high density samples were counted per subsample. Differences in cell densities relative to the untreated controls were calculated as percent control: (untreated control-treatment)/untreated control*100.

In vivo chlorophyll *a* and phycocyanin concentrations were quantified using a YSI ProDDS fluorescence-based sensor (Yellow Springs, OH, USA). For the benthic phase, growth was quantified using estimates of surface area coverage from ImageJ (Schneider et al., 2012). To quantify percent coverage of benthic mats, all beakers were photographed using a 1792 by 828 pixel resolution 326 pixels per inch (PPI) digital camera. Light and camera settings remained constant during photographing. Percent coverage of green pigments was analyzed using ImageJ. To

calculate percent coverage, bottom sediments were selected and the remaining image was cropped. The total number of pixels within the image area were determined using the measure function. Areas with a green hue were identified between 44 and 124 and selected by setting this as the color threshold in Image J. The number of pixels with a green hue were measured and percent coverage was calculated using the following equation.

$$Percent \quad coverage = \frac{Total \quad pixels}{Green \quad pixels} \times 100\%$$

2.5. Statistical analyses

To evaluate the efficacy of algaecides, cyanobacteria responses were tested for normal distribution and homogeneity of variance using Shapiro-Wilks and Brown-Forsythe tests, respectively. Algal responses that were normally distributed with homogeneous variance (i.e., chlorophyll *a*, phycocyanin and benthic coverage) were analyzed by one-way analysis of variance (ANOVA). Cell densities were normally distributed but failed the test for homogeneity of variance ($p = 0.0065$). Therefore, cell densities were analyzed using Welch's ANOVA. Dunnett's multiple comparisons test was used to identify differences relative to the untreated control and differences between single and repeat treatments. Follow-up pairwise comparisons were considered significant at $p \leq 0.05$. To assess correlations between cell densities and other cyanobacteria response measurements, Pearson correlation coefficients were calculated. Statistical analyses were conducted using GraphPad Prism version 9.1.0 for Windows (GraphPad Software, San Diego, California USA).

3. Results and discussion

3.1. Comparison of algaecides

Three of 9 sand controls had detectable concentrations of planktonic chlorophyll *a* ranging from 2 μg/L to 26 μg/L, and all other response measurements were below the detection limit. Inspection by light microscopy revealed that the algae containing the chlorophyll *a* were diatoms, with no observable cyanobacterial species present. Therefore, the sand controls provide evidence that chlorophyl *a* concentrations may provide misleading information if it is assumed that concentrations are associated with potentially HAB forming cyanobacteria. Cell density measurements confirmed that cyanobacteria had been efficiently removed from the surficial water, and there was no evidence of cyanobacteria contamination from external sources during the 14 d incubation period.

Overwintering cyanobacteria in the untreated controls germinated and grew throughout the 14 d incubation period, as indicated by increases in planktonic cell densities, pigment concentrations (chlorophyll *a* and phycocyanin), and benthic coverage. As stated previously, cyanobacteria were not detectable in the overlying (filtered) water at the initiation of the experiment. By 14 d, the average planktonic cell density was 4.8×10^5 cells/mL (± 1 SD = 3.0×10^5 cells/mL; n = 9) and would exceed KDHE's "HAB Warning" level advisory of 2.5×10^5 cells/mL, indicating that if this density of cyanobacteria was measured in situ, all human and animal contact with water should be avoided due to potential risks (KDHE, 2022b).

High variances in terms of cell densities were anticipated in this study based on previous evaluations (Calomeni et al., 2022 in review) despite carefully homogenizing sediments prior to experiment initiation. These variances may be due to heterogeneity in viable overwintering cells at the sediment-water interface as not all overwintering cells identified in sediments have the potential to germinate and transfer to the water column. Due to high standard deviations, statistical differences in terms of cell densities between the untreated controls and treatments were challenging to discern and only treatments resulting in substantial declines were identified.

Following the 14 d incubation, the average chlorophyll *a* concentration of untreated controls was 13 μg/L (± 1 SD = 5 μg/L), and average phycocyanin concentration was 4 μg/L (SD = 2 μg/L). In the sediment phase, average benthic coverage was 13% (SD = 5%) at 14 d post incubation. Percent abundance of cyanobacteria in the untreated controls included 62% *Chlorogloeopsis,* 21% *Dolichospermum,* 13% *Nostoc,* 3% *Microcystis,* and 1% *Aphanizomenon. Aphanizomenon, Dolichospermum. Microcystis,* and *Nostoc* are known to have strains that produce toxins (e.g., anatoxins, cylindrospermopsins, microcystins, nodularins and saxitoxins) and commonly form HABs (Beaver et al., 2018; Graham et al., 2008; Graham et al., 2020; Rosen and St. Amand, 2015). Growth and transfer of sediment-associated cyanobacteria to the water column in the untreated controls provide evidence of the planktonic growth potential if overwintering cells are left unmanaged within the pond.

Two of the treatments resulted in a 91% decrease in average planktonic cell densities relative to the untreated controls. These were (a) successive treatments of CuSulfate and PeroxiSolid applied 24 h apart ($p = 0.0261$) and (b) two treatments of the same algaecide, PeroxiSolid applied 24 h apart ($p = 0.0217$) (Table 3; Fig. 3). Three of the treatments yielded at least a 50% decrease in average planktonic cell densities relative to the untreated controls, although these differences were not significant. These were (a) a single treatment of PeroxiSolid, (b) successive treatments of CuAdjuvant and PeroxiSolid applied 24 h apart, and (c) the same algaecide, PeroxiLiquid 18%PAA applied twice, 24 h apart.

Successive treatments of CuSulfate and PeroxiSolid applied 24 h apart and the same algaecide, PeroxiLiquid 5%PAA and PeroxiLiquid 18%PAA each applied twice resulted in at least 50% decreases in average planktonic pigment concentrations (i.e., chlorophyll *a* and phycocyanin). Decreases in planktonic pigment concentrations (i.e., chlorophyll *a* and phycocyanin) relative to the untreated controls were evident by non-detect phycocyanin concentrations with the successive treatments of CuSulfate and PeroxiSolid applied 24 h apart and the same algaecide, PeroxiLiquid 5%PAA and PeroxiLiquid 18%PAA each applied twice.

The most effective treatments tested were two successive

Table 3
Percent control in terms of planktonic cell density (measured with Palmer-Maloney counting chamber) reduction (relative to control) following algaecide exposures and a 14 d incubation period.

Treatments[a]	Percent Control	Response Relative to Untreated Control
CuSulfate + PeroxiSolid	91	Significantly less ($p \leq 0.05$)
PeroxiSolid 2x	91	
PeroxiSolid	72	Greater than 50% decrease[b]
CuAdjuvant + PeroxiSolid	57	
PeroxiLiquid 18%PAA 2x	54	
CuChelated + PeroxiSolid	36	Relatively small decrease[b]
PeroxiLiquid 5%PAA 2x	4	
PeroxiLiquid 5%PAA	-128[c]	Greater cell density[b]
CuSulfate	-246[d]	
CuAdjuvant	-325[d]	
CuChelated	-645[d]	
PeroxiLiquid 18%PAA	-919[c]	

[a] CuSulfate = ABTM Brand Copper Sulfate Crystals; CuChelated =Cutrine-Plus®; CuAdjuvant = Cutrine®-Ultra; PeroxiSolid = GreenClean® Pro;PeroxiLiquid 5%PAA = GreenClean Liquid 5.0; PeroxiLiquid 18%PAA = GreenClean 18

[b] Not significantly different from untreated control

[c] Cyanobacterial assemblage consisted of Aphanizomenon, Dolichospermum, and Nostoc in the planktonic phase after a 14 d incubation period.

[d] Cyanobacterial assemblage dominated (84% to 98%) by Aphanizomenon inthe planktonic phase after a 14 d incubation period.

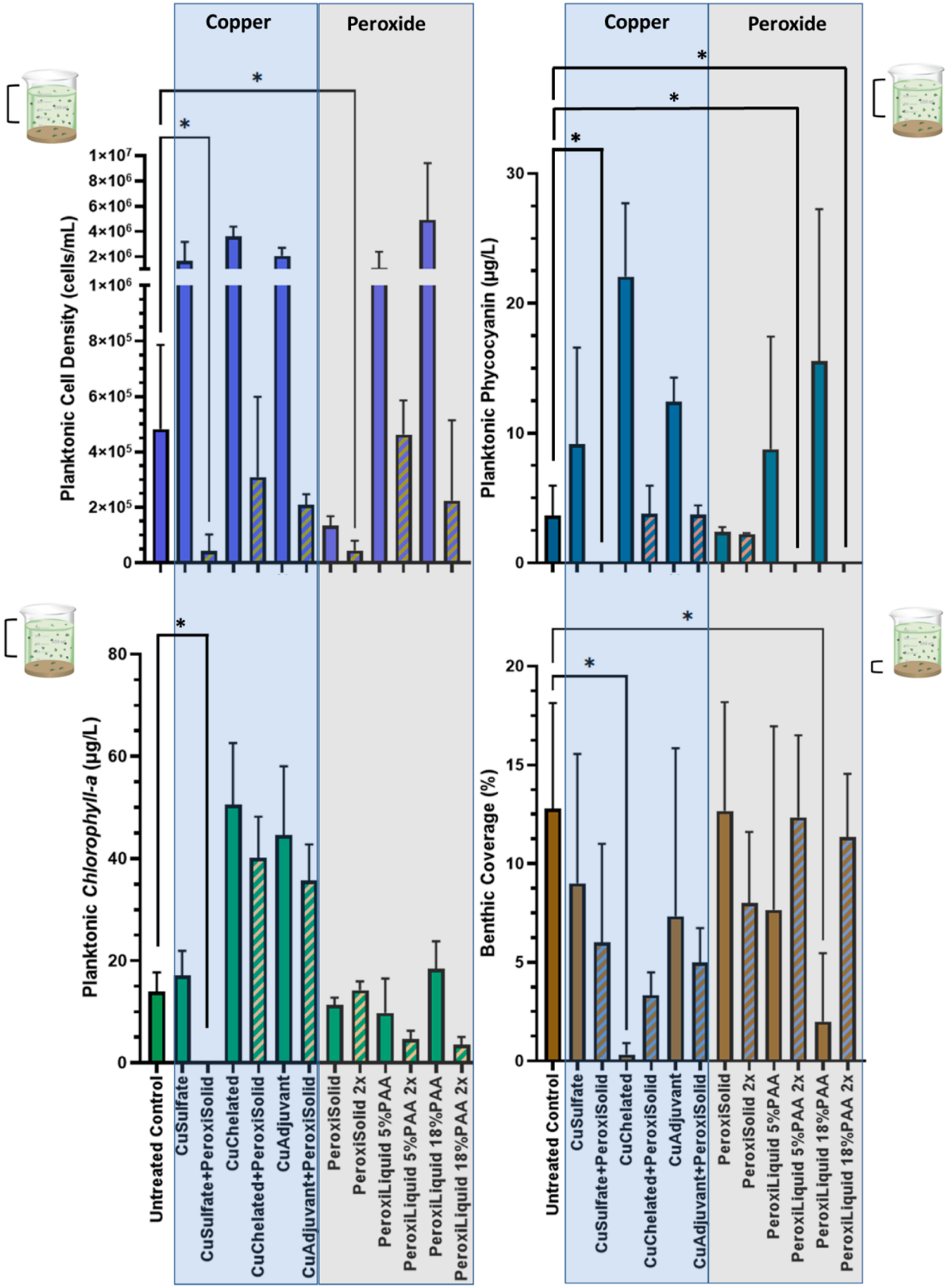

Fig. 3. Planktonic (cell density, chlorophyll *a* and phycocyanin concentrations) and benthic (percent coverage) responses of cyanobacteria from sediments containing overwintering cells following algaecide exposure and a 14 d incubation. Asterisks indicate significant differences relative to the untreated control or all replicates being below the detection limit for the response measurement. Hatched bars indicate that two treatments were applied, with the second treatment applied 24 h following the first.

applications 24 hr apart, either of CuSulfate followed by PeroxiSolid, or of PeroxiSolid followed again by PeroxiSolid (Fig. 3). Overall, PeroxiSolid was the most effective algaecide for the treatment of overwintering cells in sediments from the Gathering Pond. This is based on the observation that PeroxiSolid was effective as a single treatment and when used as the second treatment after CuSulfate, CuAdjuvant, or PeroxiSolid (applied twice) (Fig. 3). Additionally, when PeroxiSolid was applied 24 h following CuChelated, planktonic cell densities were significantly lower ($p = 0.0250$) and phycocyanin concentrations were statistically less ($p = 0.0004$) relative to CuChelated applied as a single treatment. Repeat treatments of CuSulfate or CuAdjuvant with PeroxiSolid applied after 24 h resulted in lower average planktonic cell densities relative to single treatments of CuSulfate (1.67×10^6 cells/mL for single and 4.34×10^4 cells/mL for repeat) and CuAdjuvant (2.05×10^6 cells/mL for single and 2.09×10^5 cells/mL for repeat). However, the relatively large standard deviations in cellular responses for these treatments (± 1 SD $= 1.49 \times 10^6$ cells/mL for CuSulfate and 6.57×10^5 cells/mL for CuAdjuvant) made it challenging to discern statistical differences. Nonetheless, relative differences in cell density among treatments provided beneficial insight to compare trends in algaecide efficacy.

PeroxiSolid is a sodium carbonate peroxyhydrate algaecide that dissipates into sodium carbonate and hydrogen peroxide when dissolved in water. Cyanobacteriacidal activity for this algaecide is attributed to the hydrogen peroxide (Geer et al., 2016; Geer et al., 2017). In one other study investigating efficacy of hydrogen peroxide for overwintering cyanobacteria, Jia et al. (2014) treated overwintering cells in situ (via sediment enclosures) using a 30% hydrogen peroxide solution followed by an application of rice straw in Meiliang Bay in Lake Taihu, China. Results from Jia et al. (2014) indicated that pigment concentrations were lower in treated enclosures (as compared to untreated controls) during the following spring and summer.

Single applications of CuSulfate, CuChelated, CuAdjuvant, PeroxiLiquid 5%PAA, and PeroxiLiquid 18%PAA resulted in average planktonic cell densities that were greater than the untreated controls (Fig. 3). The copper-based algaecide treatments (i.e., CuSulfate, CuChelated, and CuAdjuvant) were dominated by the cyanobacterium *Aphanizomenon*. The percent abundance of *Aphanizomenon* relative to the total number of cyanobacteria counted was 84% for CuSulfate, 98% for CuChelated, and 90% for CuAdjuvant relative to $< 1\%$ in the untreated controls, indicating that *Aphanizomenon* from the Gathering Pond may be relatively insensitive to copper-based algaecides. A possible reason for the increase in cell density for these treatments is that other competing cyanobacteria may be more sensitive to CuSulfate, CuChelated, and CuAdjuvant (Cedergreen et al., 2007). Treatments would therefore decrease densities of competing cyanobacteria and allow for the proliferation of *Aphanizomenon*. To thoroughly evaluate this hypothesis, axenic cultures of each cyanobacterium would need to be treated separately. There was a shift in algal abundance for the peroxide-based algaecides relative to the untreated control as well. Percent abundance for the single treatment of PeroxiLiquid 5%PAA was 49% *Aphanizomenon*, 45% *Nostoc,* and 6% *Dolichospermum*. Percent abundance for the single treatment of PeroxiLiquid 18%PAA was 65% *Aphanizomenon*, 31% *Dolichospermum,* and 4% *Nostoc*. In addition to shifts in dominance of competing cyanobacteria, another potential reason for the apparent increase in cell density could be due to increases in variance that can occur in populations following exposures to a stressor (Forbes, 2000). The standard deviation for the untreated control was 3.04×10^5 cells/mL relative to 1.29×10^6 cells/mL and 4.50×10^6 cells/mL for PeroxiLiquid 5%PAA and PeroxiLiquid 18%PAA, respectively.

Benthic coverage following treatments of CuChelated ($p = 0.0116$) and PeroxiLiquid 18%PAA (0.0400) were significantly less that the untreated control (Fig. 3). These results are contrary to cyanobacteria response measurements from the planktonic phase (i.e., cell density, pigment concentrations). Correlations among cyanobacterial response measurements are discussed further in the subsequent section.

3.2. Correlations between cyanobacterial response measurements and cell density

Because there are limited data measuring cyanobacteria responses that have germinated and grown from overwintering cells, there are relatively limited resources identifying informative metrics to measure responses following algaecide exposures. Therefore, data supporting appropriate measurements of these responses are necessary. Planktonic cell density is a reliable measurement of algal responses from an assemblage when there is interest in the response of a cyanobacterium or cyanobacteria, as the algae of interest can be distinguished from others on a cell-by-cell basis (Calomeni et al., 2014). For this research, planktonic phycocyanin were strongly correlated with planktonic cell density ($r = 0.89$) (Fig. 4). These data suggest that the use of a fluorescence-based sensor to measure phycocyanin in vivo could be used to detect responses of cyanobacteria that germinated from overwintering cells in sediments following algaecide exposures. This measurement is relatively rapid (i.e., seconds) and requires limited technical expertise. There may be situations in which the use of algaecides select for non-toxigenic cyanobacteria genera, and this shift in algal abundance would not be detected using phycocyanin measurements, as this pigment is contained by all cyanobacteria irrespective of the potential for toxin production. However, cell density measurements of a few subsamples could be used to identify differences in cyanobacteria abundance.

Planktonic chlorophyll *a* concentrations were not strongly correlated with planktonic cell density ($r = 0.37$) (Fig. 4). Because chlorophyll *a* is contained in all algae, this measurement does not have the resolution to distinguish cyanobacteria from other algae that are contained in "natural" samples. During identification and enumeration of cyanobacteria in the water column following treatment and a 14-day incubation period, non-target diatoms were also noted as part of the planktonic assemblage in some samples and would contribute to the total chlorophyll *a* measurement. Measurement of chlorophyll *a* concentrations could be useful for the measurement of cyanobacterial responses if cyanobacteria were the only algae present in samples.

Benthic coverage was negatively correlated with cell density ($r = -0.49$) indicating that this metric is not useful for the measurement of cyanobacteria responses in this context (Fig. 4). One possible reason for the negative relationship is that for replicates in several treatments (i.e., single treatments of CuSulfate, CuChelated, CuAdjuvant, PeroxiLiquid 5%PAA, and PeroxiLiquid 18%PAA) planktonic cell densities obstructed the sediment phase during photographing. When data from these replicates were removed from the analysis, the Pearson correlation coefficient was positive; however, still not strongly correlated ($r = 0.36$). Nonetheless, these data suggest that there is not a strong relationship between the cyanobacteria that remain in the sediment and those that can transfer to the water column in these bench-scale experiments.

3.3. Implications for Proactive Mitigation Strategies

PeroxiSolid was clearly effective at decreasing densities of overwintering cyanobacteria from Milford Gathering Pond sediments that were capable of germinating and transferring to the water column. These results provide evidence of the concentration of hydrogen peroxide that is needed to achieve a decrease in cyanobacteria inoculum from the sediment phase in situ. Treatments within Milford Gathering Pond that achieve this concentration of hydrogen peroxide at the sediment-water interface could anticipate a similar decrease in cyanobacteria inoculum from sediments. Algae associated with sediments have been successfully treated with algaecides in situ, therefore, benthic algaecide application technology is already available (e.g., weighted dropper hoses, granular algaecide formulations) (Huddleston et al., 2015; Geer et al., 2017). However, data from in situ proactive treatments to the sediment phase are needed to inform the scalability of this

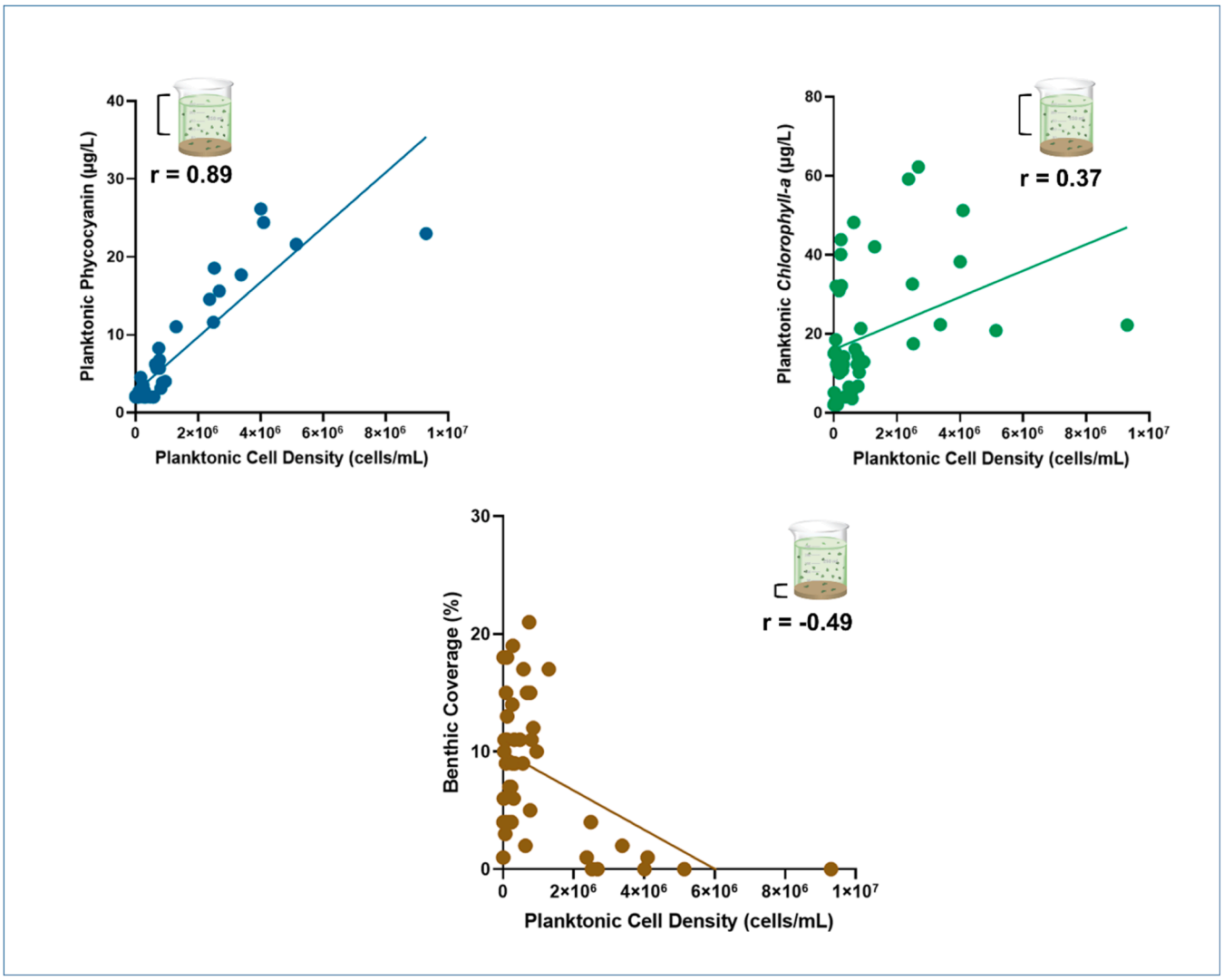

Fig. 4. Correlations between response measurements (i.e., planktonic phycocyanin and planktonic chlorophyll *a* concentration and benthic percent coverage) and planktonic cell density. Pearson's correlation coefficient (r) is indicated on each graph.

solution.

Additional uncertainties associated with proactive treatments include margins of safety for non-target species. The lack of correlation between chlorophyll *a* concentrations and cyanobacteria cell densities and presence of diatoms following treatment in this study suggests that a margin of safety (MOS) exists for some non-target algae. There have been multiple algaecide reviews published that calculate MOS for USEPA registered algaecides (Bishop et al., 2014; Geer et al., 2016; Murray-Gulde et al., 2002). These reviews include the products evaluated in this study with the exception of the liquid peroxide-algaecides (i.e., PeroxiLiquid 5%PAA and PeroxiLiquid 18%PAA). MOS are an effective metric to normalize safety data relative to concentrations that may be applied to a treatment area (e.g., area in proximity to target organism). MOS are calculated as the ratio of a toxicity value for a non-target species (e.g., LC_{50}) as the numerator and an effective concentration for a target species or maximum label concentration as the denominator. However, toxicity values for non-target species are calculated from toxicity experiments conducted at the bench scale using clean laboratory formulated water devoid of sediments. Sediments are a critical exposure modifying factor in this scenario that would presumably decrease non-target responses to algaecides (Calomeni et al., 2015). Therefore, toxicity experiments conducted using exposure chambers containing sediment would provide better estimates of non-target species sensitivities to algaecide exposures in proximity to sediments.

4. Conclusions

The purpose of this study was to generate data to inform the overall hypothesis that algaecides could be used to treat overwintering cyanobacteria in sediments and potentially delay the onset and decrease the severity of a HAB. To provide initial data for this hypothesis, bench-scale data were generated to evaluate the efficacy of six algaecides (n = 3 peroxide-based; n = 3 copper-based) as single and repeat treatments (i.e., 12 treatment scenarios). The most effective treatment scenarios based on significant declines in planktonic cell densities relative to the untreated controls, were: 1) CuSulfate followed by PeroxiSolid 24 h later, and 2) two applications of PeroxiSolid 24 h apart.

Informative measurements are needed that can distinguish responses of potentially harmful cyanobacteria to algaecide exposures from responses of other algae present in a field collected sediment containing overwintering cells. In this study, in vivo phycocyanin measured with a fluorescence-based sensor strongly correlated with cell density and therefore may provide an effective rapid response monitoring tool for in situ field monitoring. Phycocyanin measurements, along with subsampling to confirm and identify potentially harmful cyanobacteria using light microscopy, could be used within this context. These bench-

scale efficacy data provide preliminary evidence that overwintering cyanobacteria in sediment can be proactively treated prior to the formation of a HAB and will result in a lower transfer of cyanobacteria to the water column where they can pose human health, ecological and economic risks.

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CRediT authorship contribution statement

Alyssa Calomeni: Writing – original draft, Investigation, Methodology, Formal analysis **Andrew McQueen**: Writing – review & editing, Funding acquisition, Supervision, Investigation **Ciera Kinley-Baird:** Writing – review & editing, Methodology, Validation **Gerard Clyde Jr.:** Conceptualization, Resources **Grace Gusler**: Validation, Investigation, Methodology **Marvin Boyer:** Conceptualization, Resources **Elizabeth F. Smith:** Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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