

# Identification and prioritization of sites with overwintering cyanobacteria to inform preventative management of harmful algal blooms

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## ABSTRACT

Cyanobacteria causing harmful algal blooms (HABs) can overwinter in sediments as quiescent cells (akinetes or vegetative colonies) and contribute to bloom resurgences. Targeting overwintering cells in sediments for preventative management may provide a viable approach to delay onset and mitigate blooms. However, there are limited resources for this novel strategy. Given the growing global impact of HABs, the ability to identify and prioritize sites that are influenced by overwintering cells will be a critical step for preventative management. Therefore, the overall objective of this study was to identify and illustrate relevant data to support identification and prioritization of sites that contain overwintering cells with the potential to form HABs. To achieve this, sediment samples were collected from three HAB-affected reservoirs (Marion Reservoir, KS; Fort Gibson Lake, OK, and Heyburn Lake, OK) as pertinent examples. Cyanobacteria enumeration and growth potential data from incubation studies were assembled for prioritization. Overwintering cells were present in all HAB-affected reservoirs, with 85% of sites ( $n = 13$ ) containing overwintering cells in sediments and 54% of sites ( $n = 13$ ) with a planktonic growth potential producing problematic cell densities ( $> 100,000$  cells  $\text{ml}^{-1}$ ). On the basis of the weight of evidence, Marion Reservoir, followed by Fort Gibson, and last, Heyburn Lake, have the greatest potential for overwintering cells to contribute to HABs. These data indicate that a monitoring approach should consider at least two lines of evidence: 1) presence and density of overwintering cyanobacteria and 2) growth potential as informed by laboratory incubation studies to predict growth risk and prioritize locations for preventative management.

*Key words:* akinetes, monitoring, resting cells, treatment.

## INTRODUCTION

Harmful algal blooms (HABs) consisting of cyanobacteria are increasingly posing threats to inland water resources because of ecological and human health risks (e.g., toxin production, biomass, and hypoxia) (Paerl and Paul 2012, Smucker et al. 2021) and there is concomitant pressure to identify durable management solutions (ITRC 2020). This is of notable interest to the U.S. Army Corp of Engineers (USACE), which manages approximately 600 impoundments where HABs can interfere with multiple designated uses (e.g., potable water supply, fish and wildlife propagation, recreation, water quality) (Linkov et al. 2009; Brooks et al. 2016). A source and contributor to annual HAB resurgence in some water bodies are quiescent cyanobacteria cells or overwintering cells that settle to sediments at the end of the growing season (Kim et al. 2005, Kaplan-Levy et al. 2010, Cirés et al. 2013, Kitchens et al. 2018). Overwintering cells are defined in this manuscript as specialized (i.e., akinetes) and vegetative cells that enable survival under nonideal growth conditions and are located at the surface and within sediment. As such, overwintering cells are functionally analogous to a seed bank, serving as a source of viable cyanobacterial cells during the growing season.

An attractive strategy to extend the interval between HAB events and lessen the severity of blooms is to target overwintering cells in the sediments as part of a preventative management strategy (Calomeni et al. 2022). Yet, there is limited information available for developing a preventative approach. A reason for this is that cyanobacterial cells located in sediments are not readily detectable during observations *in situ* and evade most monitoring protocols (Wood et al. 2020). Additionally, there are few mesocosm or field-scale examples of preventative strategies targeting overwintering cells, as the current HAB management paradigm is primarily focused on targeting planktonic blooms after they have achieved problematic cell densities or toxin concentrations that impair water resources.

There are inherent challenges to overcome for monitoring overwintering cells in sediments that are not readily observable in the field (as compared with traditional blooms). Identification and enumeration of overwintering cells in the sediment can be achieved using particle separation techniques (e.g., dilution, density and size separation) followed by traditional microscopy (Calomeni et al. 2022). These data refer to cells that are present at the

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time of sampling and provide a line of evidence related to potential formation of HABs. However, these data may be an inaccurate indicator of viability and potential for cell growth. For example, overwintering cells may be identified that will later degrade as degradation rates may be slowed or arrested in low temperatures (Bergström et al. 2010, Gudasz et al. 2010) that are anticipated during the overwintering period. Additionally, for akinetes, timing of germination or the emergence of a new vegetative cell from the akinete envelope can vary depending on environmental conditions (Huber 1984, Rai and Pandey 1981) and make it challenging to discern growth potential on the basis of single lines of evidence (i.e., presence/absence) (Calomeni et al. 2022).

Additional lines of evidence for potential cyanobacterial viability and growth potential *in situ* can be gained from incubation studies. Incubation studies are operationally defined in this research as experiments that utilize site-collected overwintering cells contained within sediments and simulate conditions that are known to produce akinete germination and vegetative cell growth. These data can be used to evaluate overwintering cell viability under a known set of environmental conditions. A positive response from the incubation study is defined as germination of akinetes or growth of vegetative cells and transfer to the water column. Ultimately, lines of evidence from incubation studies and the enumeration of overwintering cells need to be assembled to provide a weight of evidence to inform HAB management actions including the prioritization of areas for management.

Prioritization of areas for preventative management is necessary for the USACE as HABs can affect designated water uses at multiple scales. At the local scale, effects can be limited to a cove or to a whole reservoir, with blooms affecting one or more recreational areas or water supply intakes. At the regional scale, effects can occur across multiple reservoirs within a watershed, affecting one or more authorized purposes including nonfederal water supply infrastructure. To prioritize areas for preventative management of overwintering cells, an approach that organizes separate lines of evidence (e.g., identification and enumeration data, planktonic and benthic growths from incubation studies) in an uncomplicated and coherent manner is needed so that a weight-of-evidence evaluation can be made.

We hypothesize that overwintering cells in sediments are potential sources contributing to HABs; therefore, if a management technique is utilized during the overwintering phase of the life cycle, then the subsequent timing, intensity, or duration of HABs could be made less severe. Within this context, the aim of this study was to identify an approach and relevant data needs to support identification and prioritization of sites that contain overwintering cells that have the potential to form a HAB. Sediments were collected from three historically HAB-affected reservoirs to provide pertinent examples. The specific objectives of this study were to 1) identify and enumerate overwintering cyanobacterial cells in sediment samples collected from three HAB-affected reservoirs managed by USACE, 2) measure overwintering cell growth potential and viability via presence of cells in the sediment and water column after

laboratory incubation studies, and 3) assemble enumeration and incubation study results to identify and prioritize example reservoirs on the basis of potential for HAB formation using a weight-of-evidence approach.

## MATERIALS AND METHODS

### HAB-affected reservoirs

Sediment and water samples were collected from Marion Reservoir in Kansas, and Fort Gibson and Heyburn Lakes in Oklahoma (Figure 1). All reservoirs selected are historically affected with HABs. Marion Reservoir is a 6,210-acre lake and authorized project purposes include flood control, water supply, water quality, and recreation (Linkov et al. 2006, USACE 2021c). HABs have been reported annually in Marion Reservoir since monitoring was initiated in 2003 (Clyde 2014) when the reservoir experienced a severe bloom consisting of *Aphanizomenon*, *Dolichospermum* (formerly *Anabaena*), and *Microcystis* (Clyde, personal communication). The 2003 bloom led to microcystin toxin concentrations ranging from 20 to 61  $\mu\text{g L}^{-1}$ , roughly 20 to 60 times greater than the World Health Organization's (WHO's) drinking-water guidance (1  $\mu\text{g L}^{-1}$  total microcystins) and subsequently required potable water to be transported into the community from a neighboring supply to replace the contaminated water until toxin concentrations decreased (Linkov et al. 2006).

Fort Gibson Lake is a 19,900-acre lake located in Oklahoma that experiences growths of the cyanobacteria *Eucapsis microscopica* (formerly *Chroococcus microscopicus*), *Planktolyngbya* sp., *Pseudanabaena* sp., and *Snowella* sp. Authorized project purposes include flood control, navigation, hydroelectric power generation, and fish and wildlife (USACE 2021a). Reoccurring HABs have been reported in Fort Gibson Lake in 2003, 2011, 2012, 2013, and 2017 with low or nondetect concentrations ( $< 2 \mu\text{g L}^{-1}$ ) of microcystins reported (Clyde 2014, Clyde 2018). Heyburn Lake, OK is a 920-acre lake that experiences growths of the cyanobacteria *Aphanocapsa* sp., *Chroococcus minimus*, *Raphidiopsis raciborskii* (formerly *Cylindrospermopsis raciborskii*), *Planktolyngbya* sp., and *Snowella* sp. Authorized project purposes include flood control, water supply, recreation, and fish and wildlife (USACE 2021b). Reoccurring HABs have been reported in Heyburn Lake in 2013 and 2017 (Clyde 2014, Clyde 2018).

### Sediment and site water sample collection

Sediment samples were collected from five sites at Marion Reservoir and Heyburn Lake and three sites at Fort Gibson Lake (Figure 1). Within each sample site, replicate sediment samples ( $n = 3$ ) were collected per location for a total of 39 samples. A Petite Ponar dredge was used to collect sediment samples from bottom substrates and a stainless-steel spoon was used to skim 0 to 2 cm of surficial sediment. Surficial sediment samples were enclosed in resealable plastic bags before placement in coolers containing ice. Four liters of water were collected in the vicinity of each sediment sample site via grab sampling at 0.5-m depth.

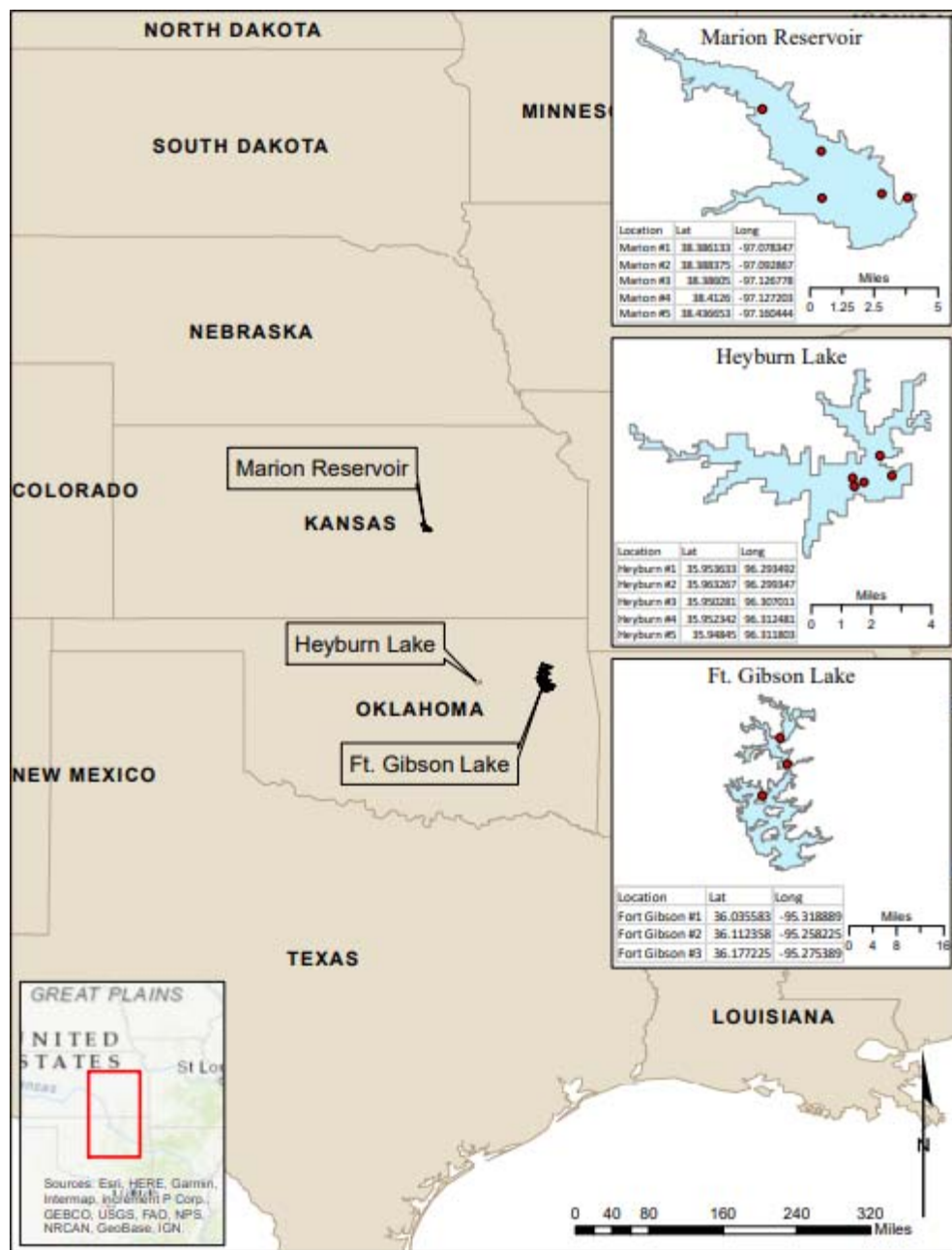


Figure 1. Map of Central United States with reservoir locations and sampling sites within each reservoir.

Water and sediment characteristics were determined using the methods and instruments outlined in Table 1.

### Identification and enumeration of overwintering cells

Approximately 1 g of wet sediment was weighed for identification and enumeration of overwintering cells. To aid in enumeration of sediment-associated overwintering cells, sediments were diluted so that cells could be visualized with a light microscope. Filtered site water was used as the diluent and was prepared with a 0.45- $\mu\text{m}$  pore size nitrocellulose filter paper to remove algae that would interfere with the enumeration of overwintering cells in

sediments. To confirm that algae were removed from the site water, site water was placed in a Palmer–Maloney counting chamber and the entire chamber (0.1 ml) was observed. Algae were not identified in the filtered site water (detection limit = 10 cells  $\text{ml}^{-1}$ ). For enumeration of cells in sediment, between 20 ml (for coarse-grain sediments) and 40 ml (for fine-grain sediments) of filtered site water was added to the wet sediment samples and were inverted to mix thoroughly. Subsamples of inverted solutions were immediately collected with a pipette and placed on a Palmer–Maloney counting chamber for enumeration. Cyanobacteria filaments, trichomes, colonies, and akinetes were enumerated using 40 $\times$  magnification with a Motic<sup>1</sup> Panthera C2

TABLE 1. METHODS FOR DETERMINING WATER AND SEDIMENT CHARACTERISTICS OF THREE HARMFUL ALGAL BLOOM (HAB)-AFFECTED RESERVOIRS.

Characteristics	Instrument/Method	Location					
		Marion Reservoir, KS		Fort Gibson, OK		Heyburn Lake, OK	
		Average ( <i>n</i> = 5)	Standard Deviation	Average ( <i>n</i> = 3)	Standard Deviation	Average ( <i>n</i> = 5)	Standard Deviation
<b>Water</b>							
pH	SevenCompact pH meter <sup>2</sup>	8.0	0.2	7.4	0.1	7.1	0.0
Conductivity ( $\mu\text{S cm}^{-1}$ )	ExStick® II <sup>3</sup>	521	69	246	21	201	2
NTU <sup>1</sup>	2100Q portable turbidimeter <sup>4</sup>	15	17	19	4	59	15
DO <sup>1</sup> ( $\text{mg L}^{-1}$ )	ProODO digital professional series <sup>7</sup>	7.2	0.5	8.5	0.2	8.4	0.3
<b>Sediment</b>							
% Solids	ASTM D2216 <sup>6</sup>	62	7	60	20	66	7
PSD <sup>1</sup>	Hydrometer <sup>7</sup>						
% Clay	—	1.5	0.5	0.5	0.2	1.1	0.5
% Silt	—	1.1	0.4	1.1	0.5	1.1	0.6
% Sand	—	97.4	0.8	87.7	17.9	97.8	0.8
% Gravel	2-mm sieve	0	0	10.5 <sup>9</sup>	0	0	0
% TOC <sup>1</sup>	Loss on ignition <sup>8</sup>	2.4	1.4	1.9	1.2	1.7	1.2

<sup>1</sup> NTU = nephelometric turbidity units; DO = dissolved oxygen; PSD = particle size distribution; TOC = total organic carbon.

<sup>2</sup> METTLER TOLEDO, Columbus, OH 43240.

<sup>3</sup> EXTECH® Instruments, Nashua, NH 03063.

<sup>4</sup> HACH®, Loveland, CO 80539.

<sup>5</sup> YSI, Yellow Springs, OH 45387.

<sup>6</sup> ASTM D2216. ASTM International.

<sup>7</sup> Bouyoucos 1962.

<sup>8</sup> Schulte 1995.

<sup>9</sup> One sample from Fort Gibson contained 31.5% gravel. The other two samples did not contain gravel. 10.5% represents the average of three samples.

brightfield transmitted light microscope. Because of the relatively low cell density in the suspended samples, the entire chamber was counted. Method detection limits were 67 cells  $\text{g}^{-1}$  for coarse-grain sediments (20-ml dilution) and 133 cells  $\text{g}^{-1}$  for fine-grain sediments (40-ml dilution).

### Incubation studies

Filtered site water was prepared by the same methods as described for overwintering cell enumeration. Site water devoid of suspended cells was used as the overlying water in the incubation study to maintain similar nutrient concentrations and ionic balance as the field locations. Sand controls were used to confirm that viable cells were not present in the filtered site water and to ensure that there was no environmental contamination (e.g., viable cells in air, beakers) during the experimental period. To create 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. For the experimental chambers, approximately 10 g of wet sediment or sand (i.e., sand controls) was weighed and placed into 250-ml borosilicate glass beakers along with 150 ml of filtered site water. To ensure that sediments were evenly distributed on the bottom of the beakers, sediments were gently stirred, if necessary.

The light intensity and temperature used during the incubation study was informed by a literature review conducted by Calomeni et al. (2022) to identify environmental conditions triggering the germination and growth of overwintering cells. Beakers were covered with a clear

polyethylene film and placed in a Darwin chamber at 25 C under continuous light. Light was provided by white (4,000 K) light-emitting diode bulbs for the duration of the study. Light intensity was measured at the mouth of the beakers and averaged 3,200 lux (standard deviation = 600 lux) or 122  $\mu\text{mol m}^{-2}\text{s}^{-1}$  (standard deviation = 5.61  $\mu\text{mol m}^{-2}\text{s}^{-1}$ ). Beakers were maintained under consistent environmental conditions for 14 d. Fourteen days was a sufficient duration to be able to discern growth, if growth was to occur, on the basis of unpublished preliminary experiments. After 14 d, beakers were removed from the experimental chambers and growth responses were evaluated in the planktonic and benthic phases.

For the planktonic phase, growth was discerned using enumeration with light microscopy as previously described and chlorophyll *a* (photosynthetic pigment) analysis. Before determining algal growth, samples were gently stirred with a glass rod to suspend any loosely associated algal cells and care was taken not to disturb sediments. For chlorophyll *a* analysis, 5- to 10-ml subsamples were collected and vacuum filtered with 0.45- $\mu\text{m}$  pore size nitrocellulose and mixed ester filters. Filters were stored at  $-20$  C before the extraction of chlorophyll *a*. Chlorophyll *a* was extracted from cyanobacteria cells using modified methods from Yepremian et al. (2017). Chlorophyll *a* concentrations were measured fluorometrically (APHA 2018) using a SpectraMax iD3 multi-mode microplate reader<sup>2</sup> and a 96-well plate. A standard calibration curve was created using a chlorophyll *a* standard<sup>3</sup> and concentrations were calculated from fluorescence values using the linear regression of the standard curve.

For the benthic phase, growth was discerned using surface-area coverage quantified with ImageJ and enumeration of sediment-associated cyanobacteria. To quantify percent

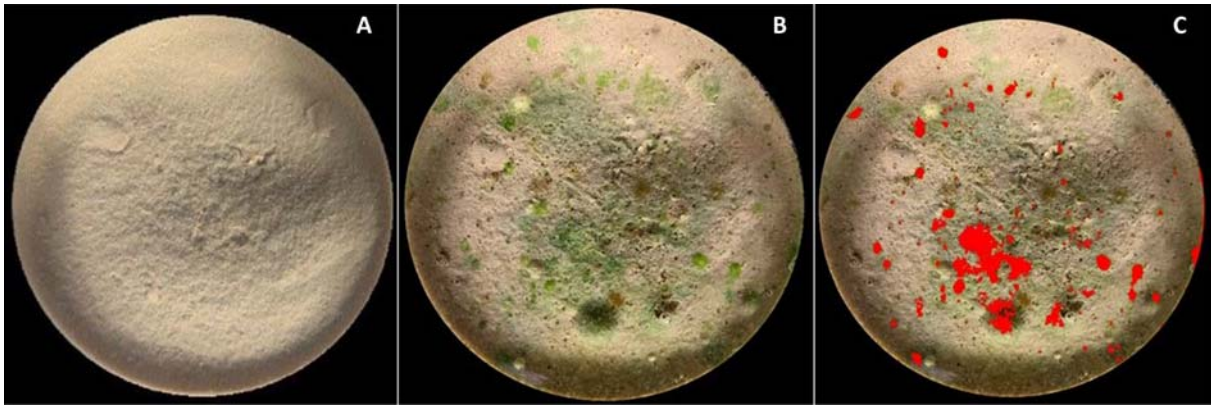


Figure 2. Percent coverage quantification using ImageJ. Macroscopic image of benthic sediments at experiment initiation (A), 14 d after initiation (B), and with green hue selected using ImageJ (C).

coverage of benthic mats, all beakers were photographed using a 1,792-by-828 pixel-resolution 326 pixels-per-inch digital camera. Light and camera settings remained constant during photographing. Percent coverage of green pigments was analyzed using ImageJ (Schneider et al. 2012) (Figure 2). To calculate percent coverage, bottom sediments were selected and the remaining image was cropped. The total number of pixels within the image area was determined using the measure function. To identify areas representing benthic algal growth, the built-in color threshold tool (using the hue-saturation-brightness color space and the default thresholding method) was used to select pixels that were primarily a green hue. The number of pixels with a green hue was measured and percent coverage was calculated using the following equation:

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

For enumeration of sediment-associated cyanobacteria via microscopy, algal mats first needed to be disaggregated using a mortar and pestle. Once the cyanobacteria were homogenized in sediments, they were enumerated using the methods previously described.

## RESULTS AND DISCUSSION

### Identification and enumeration of overwintering cells

Akinetes and sediment-associated trichomes and colonies were present at each reservoir. At Marion Reservoir, akinetes were present at all sites at relatively low densities (67 to 600 akinetes  $\text{g}^{-1}$  sediment) (Table 2). Reported akinete densities in sediments of HAB-affected water bodies range from 150 akinetes  $\text{g}^{-1}$  sediment (Legrand et al. 2017) to 36,000,000 akinetes  $\text{g}^{-1}$  sediment (Ramm et al. 2012). Cyanobacteria trichomes were present at all sites within Marion Reservoir and trichomes at sites 2 and 3 included the genus *Anabaena* (67 trichomes  $\text{g}^{-1}$ ) in the order Nostocales, having the potential to produce akinetes. Trichomes and colonies of common HAB-producing genera were present at sites 3 (*Planktothrix* and *Anabaena*) and 4 (*Microcystis*) within Marion Reservoir. Cyanobacteria densi-

ties associated with the sediment phase were generally low in Marion Reservoir and ranged from 67 akinetes and trichomes  $\text{g}^{-1}$  to 2,867 trichomes  $\text{g}^{-1}$ .

At Fort Gibson Lake, OK, overwintering cells were present at two of three sites that were sampled (Table 2). Akinetes were present at sites 1 and 2 at 133 akinetes  $\text{g}^{-1}$ . Cyanobacteria trichomes and colonies at this site included the orders Oscillatoriales and Synechococcales and do not produce akinetes nor were common HAB-forming genera. Densities of cyanobacteria colonies and trichomes were generally low and ranged from 67 colonies  $\text{g}^{-1}$  to 267 trichomes  $\text{g}^{-1}$ . No overwintering cells were observed at site 3, with a detection limit of 67 cells  $\text{g}^{-1}$ .

In sediment samples from Heyburn Lake, OK, overwintering cells were present at four of five sites (Table 2). Akinetes were present at sites 1 through 4 and densities ranged from 67 akinetes  $\text{g}^{-1}$  to 467 akinetes  $\text{g}^{-1}$ . Cyanobacteria trichomes were also present at sites 1 through 4; however, in most cases, these trichomes are not common HAB-producing genera. The exception is for site 1, where 67 trichomes  $\text{g}^{-1}$  were identified as *Anabaena* and 67 colonies  $\text{g}^{-1}$  were identified as *Microcystis*. Similar to the other reservoirs sampled, overwintering cell densities in Heyburn Lake were low and ranged from 67 cells, colonies, or trichomes  $\text{g}^{-1}$  to 467 cells  $\text{g}^{-1}$ . Overwintering cells were less than the detection limit of 133 cells  $\text{g}^{-1}$  sediment at site 5.

Overwintering cells were higher in Marion Reservoir, KS (maximum density 600 akinetes  $\text{g}^{-1}$ ) relative to Fort Gibson Lake, OK (maximum akinete density 133 akinetes  $\text{g}^{-1}$ ) and Heyburn Lake, OK (maximum akinete density 467 akinetes  $\text{g}^{-1}$ ). These data suggest that more akinetes are being produced from HAB events at Marion Reservoir and accordingly, Marion Reservoir has historically experienced more severe and frequent HABs of akinete-producing genera (i.e., *Dolichospermum* and *Aphanizomenon*) relative to the other two lakes (Linkov et al. 2006, Clyde 2014, Clyde 2018). Trichome densities were also higher at Marion Reservoir (maximum 2,867 trichomes  $\text{g}^{-1}$ ) relative to Fort Gibson (maximum 267 trichomes  $\text{g}^{-1}$ ) and Heyburn (maximum 133 trichomes  $\text{g}^{-1}$ ) lakes, indicating that greater cyanobacteria densities are associated with the sediment phase. The identification of cells from each of the sampled

TABLE 2. OVERWINTERING CELL DENSITIES IN THREE HARMFUL ALGAL BLOOM (HAB)-AFFECTED RESERVOIRS (N = 3 PER SITE).

Location	Site	Overwintering Cell Type	Algal Genera	Potential Akinete (A) or HAB Production (H) <sup>1</sup>	Density (cells, trichomes, or colonies g <sup>-1</sup> )	
Marion Reservoir, KS	1	Akinete	—	A, H	67	
		Trichome	<i>Jaaginema, Limnothrix</i>	—	1,800	
	2	Akinete	—	A, H	400	
		Trichome	<i>Jaaginema, Geitlerinema</i>	—	1,400	
	3	Akinete	—	A, H	67	
			—	A, H	133	
		Trichome	<i>Alyssophoron, Geitlerinema</i>	—	733	
			<i>Planktothrix</i>	H	200	
	4	Akinete	—	A	67	
			—	A, H	600	
		Trichome	<i>Geitlerinema, Oscillatoria, Jaaginema, Komvophoron</i>	—	2,867	
			<i>Microcystis</i>	H	200	
			<i>Microcrocis</i>	—	133	
	5	Akinete	—	A, H	400	
		Trichome	<i>Limnothrix</i>	—	1,200	
Fort Gibson, OK	1	Akinete	—	A, H	133	
		Trichome	<i>Wilmottia</i>	—	133	
	2	Akinete	—	A, H	133	
		Trichome	<i>Geitlerinema, Planktolyngbya, Alyssophoron</i>	—	267	
	3	Colony	<i>Merismopedia</i>	—	67	
		—	—	—	< 67	
		—	—	—	< 67	
	Heyburn Lake, OK	1	Akinete	—	A, H	467
			Trichome	<i>Anabaena</i>	A, H	67
<i>Geitlerinema</i>				—	133	
Colony		<i>Microcystis</i>	H	67		
		<i>Microcrocis</i>	—	267		
		—	—	—	67	
2		Akinete	—	A, H	67	
		Trichome	<i>Geitlerinema</i>	—	67	
			Colony	<i>Merismopedia</i>	—	67
3		Akinete	—	A, H	67	
		Trichome	<i>Geitlerinema</i>	—	67	
			Colony	<i>Microcrocis, Merismopedia</i>	-	333
4	Akinete	—	A, H	333		
	Trichome	<i>Jaaginema</i>	—	133		
5	—	—	—	<133		

<sup>1</sup> A = potential akinete production (Komárek and Zapomělová 2007); H = common HAB producer (Graham et al. 2008; Rosen and St. Amand 2015; Beaver et al. 2018; Graham et al. 2020).

reservoirs provides data that cyanobacteria are present and overwintering in the sediment phase. Yet, the density of viable overwintering cells that can be transmitted to the water column and form a HAB is unclear from these data. An additional line of evidence regarding overwintering cell viability can be provided by incubation studies.

### Incubation study

All reservoirs sampled had at least one site with measurable densities of planktonic cyanobacteria by 14 d after experiment initiation. All sites from Marion Reservoir and Fort Gibson Lake resulted in the transfer of sediment-associated cyanobacteria to the planktonic phase within 14 d as indicated by cell densities and chlorophyll *a* concentrations (Figure 3). In Heyburn Lake, only site 5 resulted in the transfer of benthic cyanobacteria to the planktonic phase during the 14-d study. In terms of chlorophyll *a* concentrations, all sites from Heyburn Lake remained below the detection limit of 10 µg L<sup>-1</sup>, indicating that this method was less sensitive at detecting the transfer of cyanobacteria to the planktonic phase. To ensure that

sufficient time had passed to allow overwintering cells to germinate and grow, sediment samples from this lake were retained for further evaluation. The Heyburn Lake sediments were maintained in the incubator for an additional 14 d and no notable differences were observed in terms of planktonic cell densities during this time.

In addition to cell transfer to the water column, cell densities of potential toxin-producing cyanobacteria exceeded cell-based risk thresholds, demonstrating a measurable contribution of potentially problematic algae. Average trichome densities ranged from 549 trichomes ml<sup>-1</sup> to 67,600 trichomes ml<sup>-1</sup> for sites with cyanobacteria in the planktonic phase at the end of the 14-d incubation study (Figure 3). Using the average number of cells enumerated per trichome, average cell densities ranged from approximately 66,000 cells ml<sup>-1</sup> to 1,400,000 cells ml<sup>-1</sup>. Kansas' Public Health Warning level is > 250,000 cells ml<sup>-1</sup>, indicating that cell densities are considered unsafe for people and animals (KDHE 2020). The WHO identifies cyanobacterial densities > 100,000 cells ml<sup>-1</sup> as very high risk due to potential exposures from cyanobacterial toxins (WHO 1999). Of the sites that had cyanobacteria in the

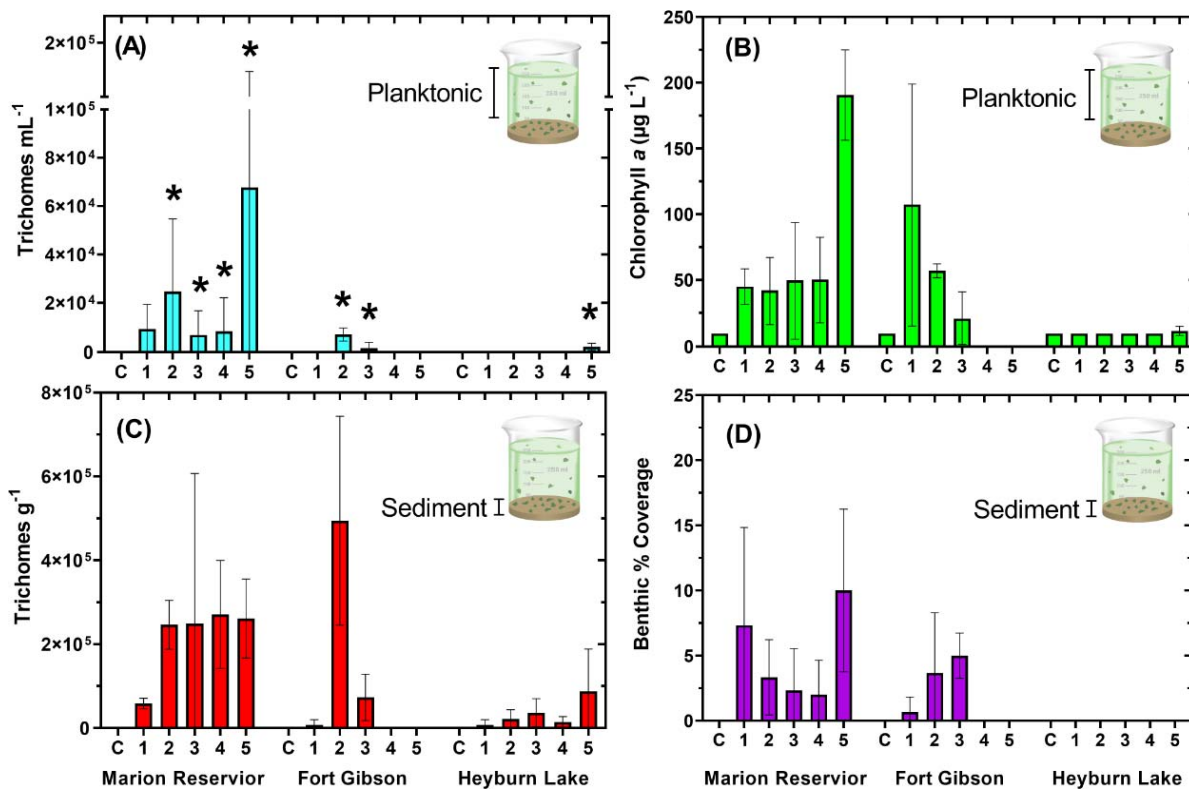


Figure 3. Fourteen-day incubation results from Marion Reservoir ( $n = 5$  sites; C = sand control), Fort Gibson ( $n = 3$  sites; C = sand control), and Heyburn Lake ( $n = 5$  sites; C = sand control). (A) Cyanobacteria trichome density (trichomes  $\text{mL}^{-1}$ ) observed in the planktonic phase. (B) Chlorophyll  $a$  concentration observed in the planktonic phase. (C) Benthic cyanobacteria trichome density (trichomes  $\text{g}^{-1}$ ). (D) Percent coverage of algal growth in sediments. Note: Asterisks in panel (A) note sites where cell densities were greater than the World Health Organization's threshold for very high risk on the basis of potential exposures to cyanobacterial toxins ( $> 100,000$  cells  $\text{mL}^{-1}$ ; WHO 1999). To distinguish sites that exceeded this threshold, trichome densities were converted to cell densities using the average number of cells per trichome of each cyanobacterium from each site.

planktonic phase, seven of nine sites exceeded the WHO cell density-based criteria of  $> 100,000$  cells  $\text{mL}^{-1}$  (Figure 3).

Of the sites that had measurable planktonic densities at 14 d, the variance in cell densities was notable. For example, within Marion Reservoir at site 5, trichome densities ranged from 6,593 trichomes  $\text{mL}^{-1}$  to 157,316 trichomes  $\text{mL}^{-1}$  (Figure 3). Large variances would be anticipated for final planktonic densities at 14 d when there are low initial cyanobacterial densities in sediments, as was observed in this study. Presumably in the incubation studies, the population was rapidly increasing (e.g., exponential growth phase). Small initial population differences would be magnified as the population continues to increase. Large differences in variance were less notable in terms of chlorophyll  $a$  concentrations, likely because this method was less sensitive. The sample from Fort Gibson at site 3 had the highest variance among replicates and ranged from 49 to 213  $\mu\text{g L}^{-1}$ .

Cyanobacteria growth was not observed in the sand controls (i.e., filtered site water and sand), demonstrating that planktonic algae were effectively removed from the aqueous phase during filtration (Figure 3). This provides evidence that the planktonic growth observed within experimental chambers containing sediments from HAB-affected reservoirs was from the sediment phase and not the filtered site water. The lack of growth in the controls serves

an additional purpose of demonstrating that there were no unintended sources (e.g., air, contamination of glassware, etc.) of viable cyanobacteria to the experimental chambers, providing further evidence that the observed growth originated from the sediment phase.

Comparison of enumerated cells (e.g., presence/absence data) and incubation study results can be used to bolster evidence for the viability of the identified cells. The identification of cells in Marion Reservoir sediments and positive incubation results provide evidence that overwintering cells at this location have remained viable and may transfer to the planktonic phase *in situ*. Similarly, at Fort Gibson, identified overwintering cells corresponded with positive incubation results at sites 1 and 2. At site 3, no overwintering cells were identified, yet cyanobacteria were present in the planktonic phase in two of three replicates. For Heyburn Lake, akinetes and overwintering cyanobacteria were present in sediments at each site except for site 5 (detection limit  $< 67$  akinetes  $\text{g}^{-1}$  sediment). However, no akinetes nor overwintering cyanobacteria detected produced measurable planktonic densities for sites 1 through 4 in the incubation study. These data suggest that the enumerated overwintering cells from Heyburn Lake sites 1 through 4 could not transfer to the planktonic phase. At site 5, where overwintering cells were less than the detection limit (133 cells  $\text{g}^{-1}$  sediment), planktonic growth was

observed in all replicates. Presumably because cells are rapidly growing within the incubation study, a low, non-detect density of viable cells may be present and lead to a planktonic growth after 14 d.

Common HAB-forming cyanobacteria that were identified in planktonic samples from Marion Reservoir at the end of the 14-d incubation study were *Aphanizomenon*, *Dolichospermum* (formerly *Anabaena*), *Pseudanabaena*, *Raphidiopsis*, and *Sphaerospermopsis* (formerly *Anabaena* and *Aphanizomenon*). In Fort Gibson, the common HAB-producing cyanobacteria identified were *Dolichospermum* (formerly *Anabaena*), *Pseudanabaena*, and *Raphidiopsis*. In Heyburn Lake, *Aphanizomenon*, and *Dolichospermum* (formerly *Anabaena*) were identified. Genera that can form akinetes include *Aphanizomenon*, *Dolichospermum*, *Raphidiopsis*, and *Sphaerospermopsis* and akinetes were presumably the source of planktonic growths in these samples. *Aphanizomenon*, *Dolichospermum*, and *Raphidiopsis* identified in planktonic samples from the HAB-affected reservoirs in Kansas and Oklahoma are ubiquitous in the conterminous United States (Beaver et al. 2018).

Planktonic measurements of overwintering cell growth, cell density, and chlorophyll *a* concentrations in overlying water provide the strongest evidence that the sediment-associated cyanobacteria are viable and have the potential to transfer to the water column. Benthic measurements were also used to determine if sediment-associated cyanobacteria are viable. In systems where sediment suspension is anticipated, these cyanobacteria (e.g., *Aphanizomenon*, *Dolichospermum*) may have the ability to transfer to the water column (Barbiero and Kann 1994, Head et al. 1999, Cires et al. 2013). All reservoirs and sites had an increase in benthic cyanobacteria densities and percent coverage after 14 d (Figure 3).

In Marion Reservoir, common HAB-producing genera that were identified in diluted sediments after the 14-d incubation included *Aphanizomenon*, *Aphanocapsa*, *Dolichospermum*, *Pseudanabaena*, and *Sphaerospermopsis* (Graham et al. 2008, Rosen and St. Amand 2015, Beaver et al. 2018, Graham et al. 2020). In addition to *Aphanocapsa*, other benthic cyanobacteria were observed such as *Leptolyngbya* and *Oscillatoria*. However, the ability of these genera to transfer to the water column and form free-floating mats is uncertain. Sediment-associated cyanobacteria from Heyburn Lake included *Aphanocapsa*, *Dolichospermum*, *Leptolyngbya*, and *Sphaerospermopsis*. With the exception of *Leptolyngbya*, all other genera commonly produce HABs in the United States. After the 14-d incubation period, cyanobacteria in Fort Gibson sediments included akinetes, *Dolichospermum*, and *Pseudanabaena*. Both algal genera *Dolichospermum* and *Pseudanabaena* commonly form HABs in the United States.

### Prioritizing regions for preventative management

To develop a guide to interpret data and prioritize reservoirs, columns representing lines of evidence, conclusions, actions, and decisions were arranged in tabular format (i.e., logic table) (Chapman 1990, Suter and Cormier 2011) (Figure 4). The three colored columns on the left side of the figure represent the different lines of evidence. The two columns on the right side represent the specific

conclusions and actionable decisions based on the lines of evidence. Green squares represent data indicating that there is limited evidence to conclude that overwintering cyanobacteria contribute to a HAB. Alternatively, red squares indicate that there is strong evidence that overwintering cyanobacteria may contribute to the formation of a HAB. The yellow squares capture scenarios between the green and red squares and could be explained by experimental errors (e.g., ineffectively removing cyanobacteria from overlying water) or differences between environmental conditions in the laboratory and field (i.e., mixing).


The tabular format was identified for use as a guide to allow for relatively rapid interpretation of study results, repeatability, and ease of communication regarding the weight of evidence analysis (Suter and Cormier 2011). Continuous numeric data for the enumeration of HAB-producing cyanobacteria and benthic and planktonic growth were converted to dichotomous data (i.e., pluses and minuses) in the table (Figure 4). This was converted so that a strong result from a line of evidence would not affect the conclusion. For example, if sediment-associated cell densities were elevated relative to all other samples but cyanobacteria were not present in the planktonic phase at the end of the 14-d incubation period, the conclusion that cells are not viable remains. In this manuscript, prioritization was conducted at the reservoir level; however, this approach could be used at different scales as well (e.g., sites within a reservoir).

On the basis of the combined results from identification and enumeration data as well as the results from the incubation study, Marion Reservoir has the greatest likelihood of benthic overwintering cells contributing to a HAB (Table 3). All sites within Marion Reservoir had akinetes, cells of common HAB-forming genera, or both. After the 14-d incubation period, benthic and planktonic growths were discerned. For Fort Gibson, at sites 1 and 2, there was also strong evidence of overwintering cells contributing to HABs. Most of the overwintering cells identified at Heyburn Lake did not transfer to the planktonic phase, indicating that monitoring would be needed to discern if mixing events suspend cells, leading to a bloom. In terms of prioritizing reservoirs for preventative management of overwintering cells, the reservoir that demonstrated the strongest evidence that overwintering cells contribute to HABs was Marion Reservoir, followed by Fort Gibson and last, Heyburn Lake.

### Research implications

The focus of this research was on benthic sources of cyanobacteria. There may be other site-specific sources of cyanobacteria in addition to benthic overwintering cells that could contribute to a HAB. A notable and documented example of an allochthonous cyanobacteria source includes the noxious HAB events that occurred in the St. Lucie Estuary in 2016 and 2018. This HAB occurred after mandated discharges into the estuary from Lake Okeechobee, which was actively experiencing a HAB (Oehrle et al. 2017, Krinsky et al. 2018, Rosen et al. 2018, Philips et al. 2020). Presumably the active HAB in Lake Okeechobee





HAB-producing Cyanobacteria Identified in Sediment	Planktonic Growth	Benthic Growth	Conclusion	Action/Decision
-	-	-	Strong evidence that overwintering cells in sediment are not the bloom source	If blooms occur, evaluate other potential sources of viable cells (e.g. inflows)
+	-	-	Identified cells are not viable	If blooms occur, evaluate other potential sources of viable cells (e.g. inflows)
-	+	-	Cyanobacteria cells were not removed from overlying water or contamination occurred	Evaluate methods for removing cells from water sample and use care to avoid contamination
-	-	+	Cyanobacteria cells in sediment are below the detection limit	Explore techniques to increase detection limit of identification and enumeration methods
+	-	+	Viable cyanobacteria did not transfer to planktonic phase without mixing	Management of overwintering cells may be necessary. Planktonic growths may be anticipated when a mixing event occurs (e.g. turnover, storm)
-	+	+	Cyanobacteria cells in sediment are below the detection limit	Explore techniques to increase detection limit of identification and enumeration methods. Management of overwintering cells may be necessary.
+	+	-	Overwintering cells are rapidly transferring to planktonic phase	Management of overwintering cells may decrease the severity and intensity of HABs
+	+	+	Strong evidence that overwintering cells are contributing to blooms	Management of overwintering cells may decrease the severity and intensity of HABs

Figure 4. Guide for interpretation of weight of evidence to identify and prioritize example reservoirs for preventative management.

served as the inoculum for the HAB event in St. Lucie Estuary. If allochthonous sources are a likely and dominant source of HABs, preventative management of sediment-associated overwintering cells may be futile. Therefore, efforts to identify potential dominant sources (e.g., river inflows) of cyanobacteria for specific sites would be an additional critical data set for identification of sites for preventative management.

Water bodies that would be candidate sites for preventative management of benthic overwinter cells would contain viable overwintering cells and have limited allochthonous sources of cyanobacteria. If a water body is identified as a candidate site for preventative management of benthic overwintering cells, monitoring of environmental

conditions can be used to refine management areas (Calomeni et al. 2022). Management areas are defined here as feasible zones for specific management techniques (e.g., chemical, physical, biological).

## CONCLUSIONS

Multiple lines of evidence are needed to support identification and prioritization of sites containing overwintering cells that have the potential to form a HAB. These lines of evidence include enumeration of akinetes and HAB-producing overwintering cells in sediment as well as benthic and planktonic cyanobacterial growth potential. The three HAB-affected reservoirs evaluated had akinetes, overwintering HAB-producing cyanobacteria, or both identified in

TABLE 3. RESULTS OF WEIGHT OF EVIDENCE EVALUATION AND PRIORITIZATION FOR PREVENTATIVE MANAGEMENT OF OVERWINTERING CELLS

Sites	Overwintering Cyanobacteria Presence	Viability/ 14-d Incubation Study Results		Conclusion	Preventative Management Priority
	Akinete or Potential HAB Producing Genera	Growth in Planktonic Phase	Growth in Benthic Phase		
<b>Marion Reservoir</b>					
1-5	+	+	+	Strong evidence that overwintering cells are contributing to blooms	<b>1</b>
<b>Fort Gibson</b>					
1, 2	+	+	+	Strong evidence that overwintering cells are contributing to blooms	<b>2</b>
3	-	+	+	Cyanobacteria cells in sediment are below the detection limit	
<b>Heyburn Lake</b>					
1-4	+	-	+	Viable cyanobacteria did not transfer to planktonic phase without mixing	<b>3</b>
5	-	+/-*	+	Cyanobacteria cells in sediment are below the detection limit	

\* Growth in the planktonic phase was detected in terms of cell densities and was below the detection limit for chlorophyll *a* concentrations (<10 µg L<sup>-1</sup>).

sediments (85% of sites; *n* = 13). Overwintering cyanobacteria were present in 100% (*n* = 5) of sites at Marion Reservoir, 66% (*n* = 3) of sites at Fort Gibson, and 80% (*n* = 5) of sites at Heyburn Lake. All sites (100%; *n* = 13) had growths of cyanobacteria in the benthic phase after a 14-d incubation study. Additionally, there was strong potential for overwintering cyanobacteria to contribute to planktonic growth. Measurable increases in planktonic cell densities were observed in 100% (*n* = 5) of sites at Marion Reservoir, 100% (*n* = 3) of sites at Fort Gibson, and 20% (*n* = 5) of sites at Heyburn Lake. Because all lines of evidence suggest that overwintering cells contribute to HAB formation in Marion Reservoir, these data suggest that this example site should be highly prioritized for preventative management, followed by Fort Gibson, and last, Heyburn Lake.

As there is limited information available for development of preventative approaches for overwintering cells, future research is needed. Future efforts should focus on identifying preventative management options (e.g., chemical, physical, or biological) to decrease the density or growth potential of overwintering cells in sediment. This study represents an initial step to inform preventative manage-

ment of overwintering cells in sediments by outlining data needs and an approach for identification and prioritization of example sites.

## SOURCES OF MATERIALS

<sup>1</sup>Motic, Kowloon Bay, Hong Kong.

<sup>2</sup>SpectraMax iD3 multi-mode microplate reader, Molecular Devices; San Jose, CA 95134.

<sup>3</sup>Chlorophyll *a* from *Anacystis nidulans*; MilliporeSigma, Burlington, MA 01803.

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