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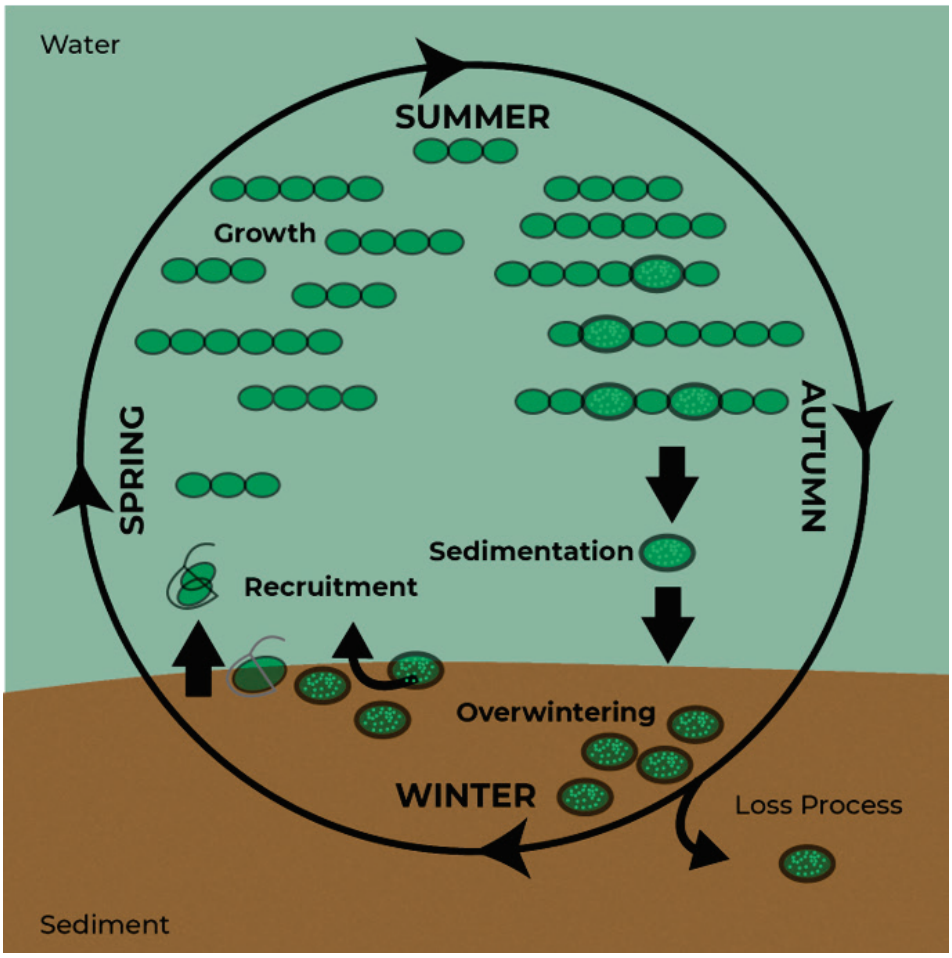
Identification and Preventative Treatment of Overwintering Cyanobacteria in Sediments

A Literature Review

Alyssa J. Calomeni, Andrew D. McQueen,
Ciera M. Kinley-Baird, and Gerard A. Clyde, Jr.

August 2022



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Identification and Preventative Treatment of Overwintering Cyanobacteria in Sediment

A Literature Review

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Abstract

Freshwaters can experience growths of toxin-producing cyanobacteria or harmful algal blooms (HABs). HAB-producing cyanobacteria can develop akinetes, which are thick-enveloped quiescent cells akin to seeds in vascular plants or quiescent colonies that overwinter in sediment. Overwintering cells produce viable "seed beds" for HAB resurgences and preventative treatments may diminish HAB intensity. The purpose of this literature review was to identify (1) environmental factors triggering germination and growth of overwintering cells, (2) sampling, identification, and enumeration methods, and (3) feasibility of preventative algaecide treatments. Conditions triggering akinete germination (light ≥0.5 μmol $m^{-2}s^{-1}$, temperature 22-27°C) differ from conditions triggering overwintering *Microcystis* growth (temperature 15-30°C, nutrients, mixing). Corers or dredges are used to collect surficial (0-2 cm) sediment layers containing overwintering cells. Identification and enumeration via microscopy are aided by dilution, sieving, or density separation of sediment. Grow-out studies simulate environmental conditions triggering cell growth and provide evidence of overwintering cell viability. Lines of evidence supporting algaecide efficacy for preventative treatments include (1) field studies demonstrating scalability and efficacy of algaecides against benthic algae, (2) data suggesting similar sensitivities of overwintering and planktonic *Microcystis* cells to a peroxide algaecide, and (3) a mesocosm study demonstrating a decrease in HAB severity following preventative treatments. This review informs data needs, monitoring techniques, and potential efficacy of algaecides for preventative treatments of overwintering cells.

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Preface

This report is part of a larger study, “Identification and Preventative Treatment of Overwintering Cyanobacterial Cells in Sediments.” This project was funded by the USACE Aquatic Nuisance Species Research Program (ANSRP) HAB Congressional Interest under Funding Account Code U4384107 and AMSCO Code 008284. Dr. Jennifer Seiter-Moser was the Technical Director for Civil Works Environmental Engineering and Sciences. Mr. Jeremy Crossland was Acting ANSRP Program Manager. The authors thank Dr. Karl Indest and Mr. Alan Katzenmeyer for reviewing an earlier version of this report.

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COL Christian Patterson was the Commander of ERDC, and Dr. David W. Pittman was the Director.

1 Introduction

1.1 Purpose

Harmful algal blooms (HABs) consisting of cyanobacteria (blue-green algae) are increasingly posing threats to inland water resources due to ecological and human health risks (e.g., toxin production, biomass, and hypoxia; Heisler et al. 2008; Paerl and Paul 2012) and subsequent loss of designated uses of those resources. There is concomitant pressure to understand impacts to the US Army Corps of Engineers (USACE) waterbodies and missions from HABs and to identify solutions (Cole 2006; Linkov et al. 2008). A myriad of mitigation strategies has been identified and implemented for controlling HABs (Herman et al. 2017), including the use of US Environmental Protection Agency (USEPA)-registered algaecides to treat planktonic blooms to achieve rapid control. Yet, a fundamental challenge associated with algaecide-based treatments to planktonic HABs is the lack of treatment durability for preventing re-occurring blooms. Therefore, there is interest in using algaecides in a more strategic and preventative manner by treating overwintering cells in sediments to decrease the source of cyanobacterial cells in a waterbody. However, this approach is novel and there are numerous uncertainties that need to be addressed prior to implementation. Therefore, to decrease uncertainties and inform the feasibility of a preventative treatment approach, this study reviews existing information on (1) environmental factors that may trigger germination and growth of cyanobacterial cells in sediments, (2) sampling, identification, and enumeration methods for overwintering cells in sediments, and (3) algaecide treatment approaches applicable to overwintering cells in sediments to prevent or minimize planktonic growth of HABs. This literature review provides data to support the use of USEPA-registered algaecide for the preventative treatment of overwintering cells. Additionally, information gained from this will inform site selection criteria for field trials to be conducted during the planned future phases of this project (USACE ANSRP “Identification and Preventative Treatment of Resting and Overwintering Cyanobacterial Cells in Sediments”).

1.2 Background

HABs are defined in this technical report (TR) as visibly dense growths of planktonic cyanobacteria posing risks to ecological receptors, humans, or both and limit the intended uses of the water resource. Early detection and management (i.e., mitigation) of HABs in water resources is critical to decrease risks efficiently and effectively to both humans and ecosystems. One commonly employed tactic for management of HABs is chemical treatment with USEPA-registered algaecides. When used proactively and strategically, algaecide applications can mitigate cell densities of HABs in relevant timescales (within days) and restore the intended uses of the water resources. Given what is known about the life cycles of cyanobacteria, there may also be an opportunity to use these products in a preventative manner.

Terminology

Overwintering Cells
General term to describe specialized cells (i.e. akinetes) or vegetative cells that enable survival under non-ideal growth conditions

Types of Overwintering Cells

Akinetes
Specialized quiescent cells formed by cyanobacteria under the order Nostocales. *Akinetes are surrounded by a thick envelope and contain greater concentrations of energy storage molecules relative to vegetative cells*

Vegetative Cells
Sediment associated colonies of cyanobacterial "resting" cells that can overwinter and remain viable for the next growing season. Common overwintering vegetative cells are under the order Chroococcales *and* Synechococcales

Overwintering cells are defined as cells that enable survival under non-ideal growth conditions (e.g., colder temperatures in the winter). Overwintering cells can take various forms depending on the cyanobacterium. Cyanobacteria from the order Nostocales form overwintering cells called akinetes - a specialized quiescent cell that precipitates to sediments when ambient conditions become unsuitable for growth (i.e., fall and winter), essentially creating a "seed bed" from which planktonic blooms are recruited the following year (Cirés et al. 2013). A common HAB-producing genus, *Microcystis*, can similarly survive unsuitable growth conditions through the development of overwintering colonies that develop protective mucilage around cells (Reynolds et al. 1981; Cirés et al. 2013). Recruitment of these cells to surface waters has repeatedly been identified as a contributing factor to algal blooms in warmer seasons (Kim et al. 2005; Kaplan-Levy et al. 2010; Kitchens et al. 2018). However, the relative contribution of germinated akinetes and survival and proliferation of overwintering colonies to summer algal blooms may vary from site to site.

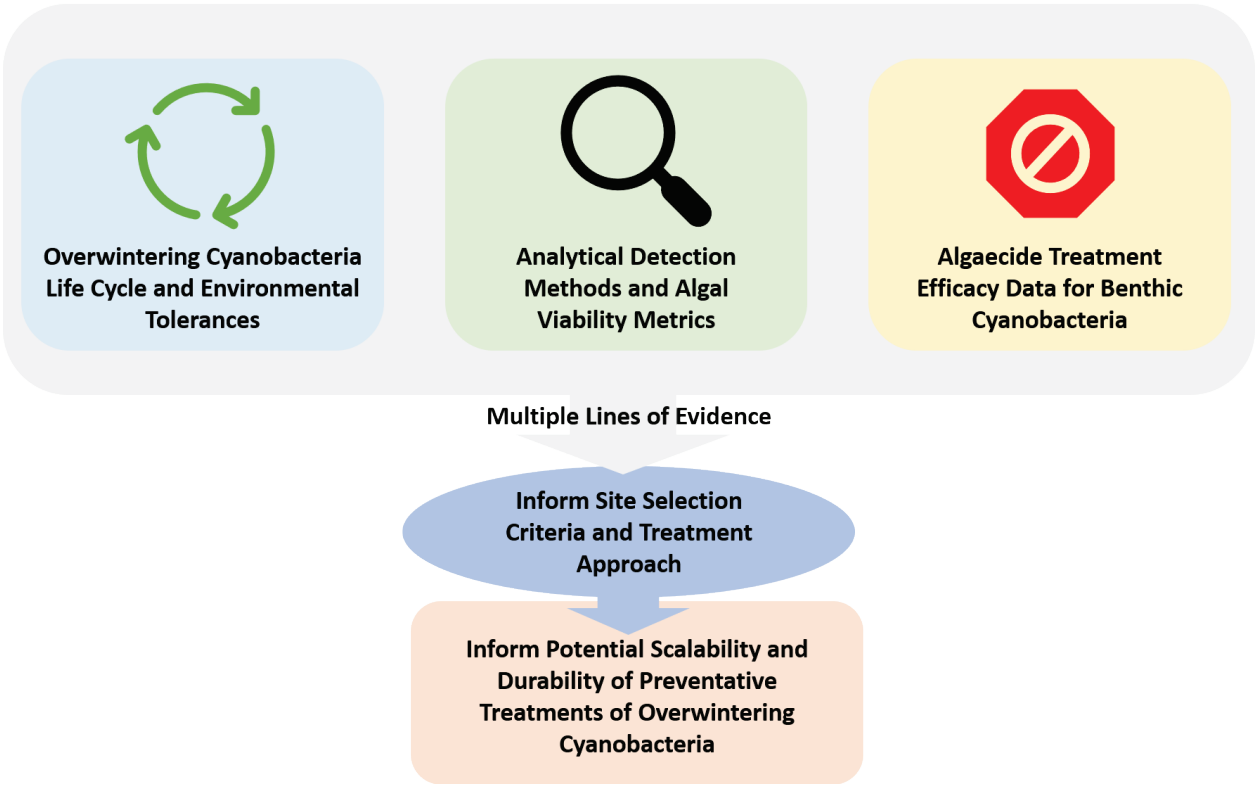
Water bodies that would be candidate sites for preventative treatments of overwintering cells would experience seasonal or recurring HABs that

develop rapidly and intensely. Early year preventative treatments using algaecides (e.g., peroxide-based) could be strategically designed to mitigate viable overwintering cells prior to germination and growth of vegetative cells, potentially minimizing the biomass and severity of HABs that form later in the year (Chen et al. 2016). In turn, this should also decrease the frequency of algaecide treatments and/or total magnitude (volume and scale) necessary for treatments later in the peak growing season. If early detection and preventive treatment of resting cells are effective, substantial value is added by decreasing severity of bloom events, human health risks, costs associated with HAB mitigation efforts, and algaecide treatment costs.

1.3 Objectives

Specifically, the objectives of this review were to (1) document life cycle information of overwintering cells in terms of reported sediment concentrations and environmental tolerances, (2) identify predominate analytical detection methods for identification and quantification of overwintering cells from sediments; and (3) review relevant data on algaecide applications to benthic overwintering cells. This information will provide insight to the feasibility of preventative treatment strategies and site selection criteria needed to identify candidate treatment areas (Figure 1).

Figure 1. Approach for literature review to inform preventative treatment of overwintering cyanobacteria.



2 Freshwater HABs of Interest

2.1 HAB genera that produce akinetes and overwintering vegetative cells

HABs often consist of numerous co-occurring cyanobacterial genera (Graham et al. 2008; Rosen and St. Amand 2015) that span different phylogenetic orders, ranging in terms of the type of overwintering cells and potential risks from toxin production. Generally, many freshwater HAB species can produce liver toxins (e.g., cylindrospermopsins, microcystins, and nodularins) and neurotoxins (e.g., anatoxins and saxitoxins). Specifically, the focus of this review was on genera that (1) are problematic due to toxin production and cell density, (2) have a portion of their life cycle in sediments, (3) can proliferate in the sediment and transfer to the planktonic zone, and (4) are often problematic in US lakes and reservoirs.

Algal genera considered common contributors to problematic blooms in freshwater systems can be subdivided into three different orders (Table 1): (1) Chroococcales, (2) Nostocales, and (3) Synechococcales. In an analysis of the 2012 USEPA National Lakes Assessment survey, genera from the order Nostocales were ubiquitously distributed throughout the conterminous US (Beaver et al. 2018). *Microcystis* of the order Chroococcales was distributed in the eastern US and genera from the order Synechococcales were not reported. Cyanobacteria of the orders Chroococcales and Synechococcales can remain as vegetative cells in colonies associated with the sediment phase (Tsuijimura et al. 2000) during non-ideal growth conditions (e.g., colder temperatures in the winter). Whereas cyanobacteria of the order Nostocales can form specialized cells, termed akinetes.

Most of the published literature regarding overwintering vegetative cells has been focused on the algal genus *Microcystis*. Although overwintering cells of *Microcystis* are not a distinct cell type from vegetative cells, some seasonal differences have been discerned in terms of density. It has been hypothesized that a decrease in gas vessel (i.e., aerotope) size and an increase in glycogen concentration results in denser *Microcystis* cells that sink to the sediments (Reynolds and Walsby 1975; Kromkamp and Mur 1984) at the end of the growing season.

Similar to *Microcystis*, *Woronichinia* also contains aerotopes and may regulate buoyancy in a comparable manner. *Woronichinia* is another genus that can produce toxins and is commonly identified in HABs (Graham et al. 2008, 2020). Populations of the potential toxin and HAB-producing genera, *Aphanocapsa* and *Pseudanabaena*, have been identified in planktonic and benthic environments. *Aphanocapsa* and *Pseudanabaena* can be associated with the mucilage of *Microcystis* in HABs (Sedmak and Kosi 1997; Vasconcelos et al. 2011; Kinley-Baird et al. 2020).

Table 1. Genera, overwintering cells and potential toxins of common HAB-producing cyanobacteria (citations for common HAB-producing cyanobacteria: Graham et al. 2008; Rosen and St. Amand 2015; Beaver et al. 2018; Graham et al. 2020).

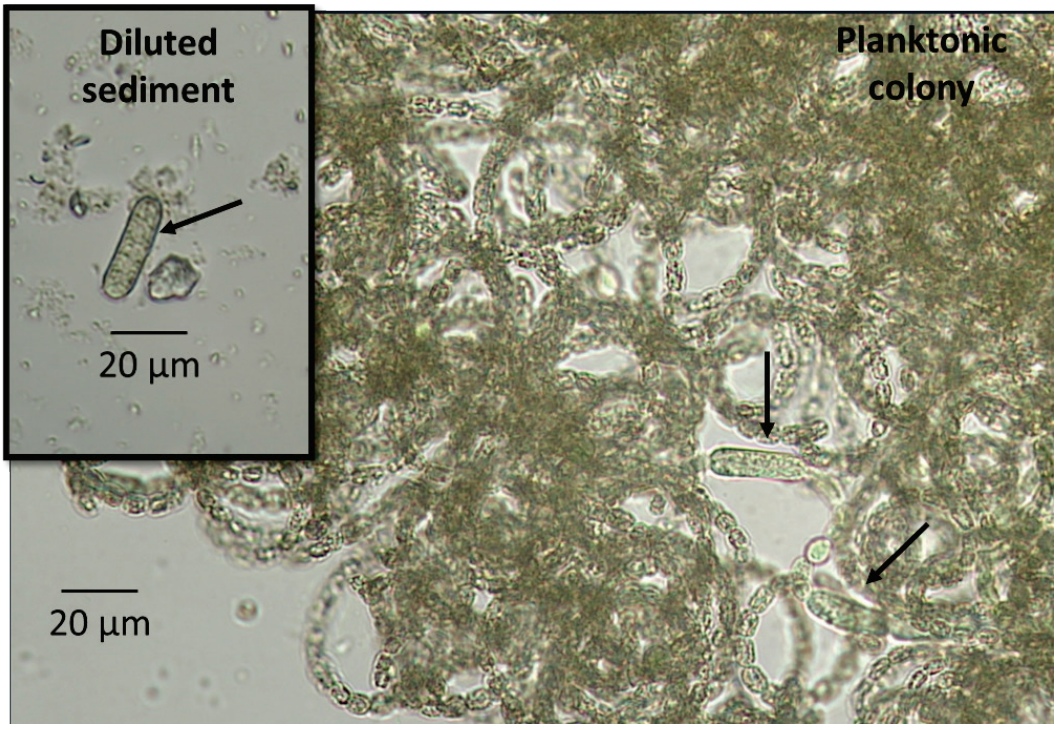
Order	Genus	Potential Toxin Type	Overwintering Cell Type	Reference[3]
Chroococcales	*Microcystis*	Liver	Sediment-associated colonies	Preston et al., 1980; Reynolds et al., 1981; Kitchens et al., 2018
Nostocales	*Anabaenopsis*	Liver	Akinete	Komárek 2010
	Aphanizomenon	Liver, Neuro	Akinete	Komárek 2010
	Cuspidothrix	Neuro	Akinete	Komárek 2010
	Cylindrospermopsis	Liver	Akinete	Komárek 2010
	Dolichospermum[1]	Liver, Neuro	Akinete	Wacklin et al., 2009
	Gloeotrichia	Liver	Akinete	Komárek and Marěs 2012
	Nodularia	Liver	Akinete	Komárek and Marěs 2012
	Nostoc	Liver	Akinete	Rajaniemi et al. 2005
	Raphidiopsis	Liver, Neuro	Akinete	Komárek 2010
	Sphaerospermopsis[2]	Liver, Neuro	Akinete	Komárek and Marěs 2012
Synechococcales	*Aphanocapsa*	Liver	Benthic and planktic populations[4]	Komárek 2003
	Pseudanabaena	Liver	Benthic and planktic populations[4]	Komárek 2003
	Woronichinia	Liver	Sediment-associated colonies	Trimbee and Harris 1984; Head 1999

[1]formerly planktonic species of *Anabaena*
[2]formerly *Anabaena* and *Aphanizomenon*
[3]Reference associated with documented overwintering cell type
[4]Planktonic and benthic populations have been observed. The extent that benthic populations overwinter and transfer to the planktonic phase has not been reported in the literature identified during this review.

Contrary to genera of the orders Chroococcales and Synechococcales, a distinct cell type is formed for genera of the order Nostocales (e.g., *Anabaenopsis*, *Dolichospermum* [formerly *Anabaena*], and *Raphidiopsis*) during non-ideal growth conditions (e.g., colder temperatures in the winter). These specialized cells are termed akinetes and are interspersed

among vegetative cells within trichomes (chains of cells) (Figure 2). These specialized cells differ from vegetative cells in that they are larger (can be ten times larger, Adams and Duggan 1999) and denser; they contain more nucleic acid (Sukenik et al. 2012), glycogen, and cyanophycin (Sutherland et al. 1985) than vegetative cells. An added difference is that akinetes are surrounded by a thickened surficial envelop (Fay 1988). It is hypothesized that the added energy storage molecules (i.e., glycogen and cyanophycin), and nucleic acid allow the cells to rapidly divide when conditions become suitable for growth (e.g., beginning of growing season); while the thickened cell covering provides protection from unfavorable environmental conditions.

Figure 2. Akinetes in planktonic *Dolichospermum* (formerly *Anabaena*) and a diluted sediment sample. Arrows point to akinetes.



To inform viability of preventative treatment approaches, environmental factors influencing the life cycle (e.g., germination) and proliferation of these HAB genera are reviewed and discussed in the following section.

3 Environmental Tolerances

Understanding environmental tolerances of HAB genera of interest are critical to inform aspects of when, where, and how to monitor within a waterbody. Additionally, these data can inform site-selection criteria for management (e.g., when, where, and how to treat in a waterbody) and inform potentially effective treatment options. It should be noted that these data are intended to be general guidelines and not "universal triggers," as many aspects of cyanobacterial emergence and proliferation are not yet fully understood.

3.1 Recruitment of overwintering cells to the water column

Favorable conditions trigger the germination of akinetes (order Nostocales) or growth and resuspension of vegetative cells that overwintered in the sediment (orders Chroococcales and Synechococcales). Understanding the environmental conditions that are "favorable" for germination or growth may inform the appropriate timing and areas for preventative treatments. Additionally, "favorable" conditions may be limited to certain areas within a water body (e.g., photic zones) and can be used to locate "seed beds" that may contribute to bloom events.

Predominate environmental factors that may influence cyanobacterial growth include light, temperature, nutrients, and oxygen. Specific ranges of these factors that are favorable are often informed by data developed in a laboratory setting where they can be readily altered using appropriate replication and controls. Additionally, each environmental factor can be altered separately in the laboratory setting so experiments are unconfounded. For example, when evaluating the impacts of light on akinete germination, temperature can be held constant, and the same nutrient sources can be used for all treatments. In Tables 2-7, environmental factors confounded in the experiment are noted. Confounded in this context refers to evaluating more than one environmental factor at a time.

For algal genera that produce akinetes, germination as an algal response is readily apparent by visualization of a germling from the envelop of the akinete. Alternatively, for vegetative cells that overwinter in the sediment, viability as the presence of a vegetative cell remains uncertain. For example, intact *Microcystis* cells may be discerned from sediments

collected in the winter. These cells may have remained intact because degradation of the cellular material was slowed in the cold and may later degrade. Care is therefore needed in the interpretation of vegetative cell responses. Multiple measurements of algal viability over a series of times may be necessary to provide defensible data that the algal population is growing or declining. As there are fundamental differences between akinetes and overwintering vegetative cells, the following sections are subdivided by cell types.

3.2 Light and photic zones

There is consensus among the published literature that light is an important driver for the germination of akinetes (Yamamoto 1976; Rai and Pandey 1981; Huber 1985; Sutherland et al. 1985; Kim et al. 2005) (Table 2). Most of the data that demonstrated the importance of light as a driver for the germination of akinetes are from laboratory grow out studies (Yamamoto 1976; Rai and Pandey 1981; Huber 1985; Sutherland et al. 1985; Kim et al. 2005). In Kim et al. (2005), results of a laboratory grow out study were supported by field observations demonstrating that light intensities necessary for germination in the laboratory also closely correlated with germination in lake sediments. No germination or low (6%) germination percentages occurred in darkness (Yamamoto 1976; Huber 1985; Kim et al. 2005). Between 20% and 96% germination occurred at 1,200 lx and 1,400 lx, respectively during continuous irradiation for 4 to 5 days (Yamato 1976; Rai and Pandey 1981). For context, the illuminance measured in daylight can range from 100 lx on an overcast day to 130,000 lx in direct sunlight (Hänel et al. 2017) and depends on latitude and season. In terms of light expressed as specific wavelengths used for photosynthesis or photosynthetic photon flux densities (PPFD), approximately 3% to 90% germination occurred at PPFDs of 0.5 – 80 μmol $m^{-2}s^{-1}$ (Huber 1985; Fay 1988; Kim et al. 2005). A PPFD of 0.5 μmol $m^{-2}s^{-1}$ is relatively low compared to the recommended PPFD of 86 μmol $m^{-2}s^{-1}$ (illuminance of 4,306 lx) by the USEPA for algal growth studies in the laboratory (USEPA 2002).

To translate responses of akinetes to light from these laboratory experiments to the field, attenuation needs to be considered. Attenuation refers to the decrease in light energy in water as water depth increases due to adsorption and scattering. The laboratory experiments reviewed were conducted in experimental chambers with relatively small water volumes (i.e., μl to ml). Therefore, light attenuation would be relatively low as the

water column in a small volume of water is shallow. Comparable illuminances and PPFDs measured at the sediment-water interface where akinetes are located should result in comparable responses as those observed in the laboratory. This contrasts with illuminances and PPFDs measured at the air-water interface because light would be attenuated prior to reaching the overwintering cells. To measure relevant illuminances and PPFDs resulting in the germination of akinetes, light measurements should be determined at the sediment-water interface.

Data from the reviewed laboratory studies suggest that in the field, multiple days in a row of low light may be sufficient to induce germination of akinetes. Illuminances and PPFDs measured at the sediment-water interface can be compared to light that resulted in akinete germination in the laboratory to identify areas within water bodies with the potential to result in germination of akinetes. Areas within the water body remaining in the dark would have a low potential for the germination of akinetes.

For overwintering cells of *Microcystis*, PPFDs from 10 μmol $m^{-2}s^{-1}$ to 100 μmol $m^{-2}s^{-1}$ and illuminance from 600 lx to 1,800 lx had negligible impacts on overwintering cell growth (Table 3). The studies that evaluated responses of overwintering *Microcystis* cells to light used different measurements to indicate that *Microcystis* cells were viable. Of the studies evaluated, responses measured on multiple days and demonstrated changes in cell density over time would provide defensible evidence of shifts in algal growth; these studies were Reynolds (1981), Mission and Latour (2012), and Borges et al. (2016). The results of these three studies support that differences in light within the specified ranges have little impact on overwintering cell viability as there were no significant changes in responses or small changes (1.4 to 1.1 times greater cell density) were observed relative to dark controls. Presence of algal cells measured at one time (Brunberg and Blomqvist 2002) and photosynthetic activity (Fallon and Brock 1981) provide less convincing evidence of shifts in cell viability and were not used to inform triggers for growth of overwintering *Microcystis* cells.

Table 2. Akinete germination and responses to various light conditions.

Algal Genera	Light Source	Light Cycle	Light Intensity	Duration (days)	Light Measurement Unit	Percent Germination	Study	Reference
Anabaena	incandescent lamp	continuous	1,200 lx	4	illuminance	20	Laboratory grow out	Yamato 1976
Anabaena	incandescent lamp	continuous	1,400 lx	5	illuminance	96	Laboratory grow out	Rai and Pandey 1981
Nodularia	cool white fluorescent	ns	0.5-80 µmol $m^{-2}s^{-1}$	5	photosynthetic photon flux density	43-90	Laboratory grow out	Huber 1985
Nostoc	warm white	ns	8,000 lx[d]	2	illuminance	germination observed, percent not specified	Laboratory grow out	Sutherland 1985
Dolichospermum	lamp with filters to exclude certain wavelengths	continuous	45 µmol $m^{-2}s^{-1}$	3	photosynthetic photon flux density	2.8-10.7	Laboratory grow out	Fay 1988[c]
Dolichospermum	ns	12h:12h	5 -30 µmol $m^{-2}s^{-1}$	6	photosynthetic photon flux density	45-50	Laboratory grow out and comparison with observations within lake	Kim et al., 2005
Nodularia	ns	ns	40 and 100 µmol $m^{-2}s^{-1}$[a]	8[b]	photosynthetic photon flux density	1 (highest salinity evaluated) - 50	Laboratory grow out	Myers et al., 2010[c]

[a] Significantly greater germination observed at 100 µmol $m^{-2}s^{-1}$ relative to 40 µmol $m^{-2}s^{-1}$

[b] Germination measured at the first-time interval (3 days)

[c] Light and other environmental factors were altered within the same treatments. In addition to light, nitrogen sources and culture media were altered within the same treatments in Fay 1988. In addition to light, salinities and nutrients were altered within the same treatments in Myers et al., 2010.

[d] luminance was measured at the surface of the experimental chamber. All other studies did not specify where light was measured.

Table 3. Overwintering *Microcystis* responses to various light conditions.

Light source	Light Cycle	Light Intensity	Duration (days)	Light measurement unit	Algal Response measurement	Algal Response	Study	Reference
ns	ns	<1,000 lx	ns	illuminance	Growth	Growth observed. Magnitude of growth not specified.	Review paper	Reynolds and Walsby 1975[ab]
incandescent bulbs	ns	100 µmol $m^{-2}s^{-1}$	0.4	photosynthetic photon flux density	Uptake of radiolabeled carbon during photosynthesis measured as disintegrations min^{-1} of ^{14}C (DPM)	DPM 2.2 times greater than dark and low light (26-75 µmol $m^{-2}s^{-1}$) treatments	Cells retained on filter paper	Fallon and Brock 1981[a]
fluorescent illumination	continuous	600 to 1,800 lx	17 and 38	illuminance	colony density (colonies/mL)	No impact on magnitude of colony density[c]	Laboratory grow out	Reynolds 1981
ns	12h: 12h	30 µmol $m^{-2}s^{-1}$	120	photosynthetic photon flux density	Cell density	Cell densities 1.6 times less than dark treatments	Laboratory survival	Brunberg and Blomqvist 2002 [a]
ns	ns	10µmol $m^{-2}s^{-1}$	8[d]	photosynthetic photon flux density	Percent recruitment (aqueous cell density/benthic cell density x 100%)	No impact on percent recruitment (0.07% - 0.75%)	Laboratory grow out	Misson and Latour 2012
ns	12h: 12h	50-100 µmol $m^{-2}s^{-1}$	12	photosynthetic photon flux density	Cell density at surface of culture container	Cell density 1.4 to 1.1 times greater than dark and low light treatments[e]	Laboratory grow out	Borges et al., 2016
ns	12h: 12h	50-100 µmol $m^{-2}s^{-1}$	12	photosynthetic photon flux density	Cell density at surface of culture container	No impact on magnitude of increase in cell density [e]	Laboratory grow out	Borges et al., 2016

ns = not specified
[a] Responses of *Microcystis* were not discerned over the course of multiple days.
[b] Unpublished data referenced in this review
[c] Responses of *Microcystis* in the light treatment occurred 21 days earlier than the dark treatment.
[d] Greatest recruitment observed on day 1 of the experiment. Sediments were collected from the field post-bloom and stored in the dark at 4°C for a few days prior to experiment initiation.
[e] Sediments were collected from "near-shore" and "mid-lake". Light impacted cell densities in laboratory grow out studies for the "mid-lake" sample and not the "near-shore" sample. Cell density from "mid-lake samples were low at all photosynthetic photon flux densities evaluated (<1,000 cells/mL).

3.3 Temperature

Germination of akinetes occurred over a wide range of temperatures (5°C to 35°C); however, the reviewed data indicate that maximum germination occurred at 22°C to 27°C for most cyanobacterial genera investigated (e.g., *Nodularia*, *Anabaena*, and *Dolichospermum*) (Table 4). The exception to

this temperature range occurred in germination of *Dolichospermum* akinetes from a Korean reservoir which had maximum growth at approximately 10°C in the laboratory and at comparable temperatures in the field (Kim et al. 2005). Although there may be some cyanobacteria suited for lower temperatures (i.e., 5°C), these results suggest that for multiple genera, the maximum germination of akinetes would be induced when temperatures at the sediment-water interface reach 22°C to 27°C for 3 to 5 days consecutively.

These data indicate that temperature measurements at the sediment-water interface could be used to inform locations and timing of potential akinete germination. For stratified water bodies, temperatures at the sediment-water interface may limit germination of akinetes below the thermocline as temperatures would remain low throughout the year. For context, minimum and maximum lake water temperatures in the United States range from 4°C to 38°C with an average of 19°C from May to October (USEPA 2007). Temperatures resulting in the maximum germination of akinetes (22°C to 27°C) would occur during the spring or summer in surface waters across the US (USGS 2020).

The growth of *Microcystis* overwintering cells was observed over a wide range of temperatures (4°C to 35°C; Table 5). In Yang et al. (2020), the greatest growth rate of *Microcystis* occurred at temperatures between approximately 20°C and 30°C in the laboratory and was confirmed in the field. Borges et al. (2016) also observed rapid growth rates at temperatures ranging from 16°C to 25°C. Temperatures from 15°C to 18°C have been associated with initial bloom appearance in the field (Reynolds and Walsby 1975). These data indicate that *Microcystis* can grow at temperatures throughout the year in the US. More rapid growth rates at temperatures from approximately 15°C to 30°C may be associated with surficial blooms.

Table 4. Temperature influences on akinete germination in laboratory grow out studies.

Genera	Temperature Range for Germination (°C)	Temperature for Maximum Germination (°C)	Maximum Percent Germination	Duration (days)	Study Type	Reference
Anabaena	20-35	27	20	4	Laboratory grow out	Yamamoto 1976
Nodularia	12-25	22	65-85	5	Laboratory grow out	Huber 1985
Dolichospermum	20-28	22	30-50	ns	Laboratory grow out	Fay 1988
Dolichospermum	5-15	10	24-50	6	Laboratory grow out and comparison with observations within lake	Kim et al., 2005
Dolichospermum	10-25	25	35-80	3	Laboratory grow out	Park et al., 2018

ns= not specified

Table 5. Responses of overwintering *Microcystis* to temperature.

Temperatures evaluated (°C)	Temperature for maximum response (°C)	Response measurement	Magnitude of response	Duration (days)	Study Type	Reference
NA	15-18	*Microcystis* bloom appearance	NA	NA	Review	Reynolds and Walsby 1975
5-37	25-30	Uptake of radiolabeled carbon during photosynthesis measured as disintegrations min^{-1} of ^{14}C (DPM)	DPM 2 to 4 times greater than lowest temperature (5°C)	0.4	Cells retained on filter paper	Fallon and Brock 1981[a]
4 and 8	8	Percent dividing cells (%)	Greater percent of population dividing at different time point throughout the day at 8°C	1	Laboratory grow out	Latour et al., 2004[a]
4-17	NA	Percent recruitment (aqueous cell density/benthic cell density x 100%)	No differences in percent recruitment among temperatures evaluated (0.29-0.41%)	8[b]	Laboratory grow out	Misson and Latour 2012
4-25	NA	Cell density at surface of culture container	No differences in cell density magnitude among temperatures evaluated [c]	12	Laboratory grow out	Borges et al., 2016
13-35	21-31	Growth rate	1.0 μd^{-1}	180	Laboratory	Yang et al., 2020
12-34	24-28	Aqueous phycocyanin concentration	400 µg/L of phycocyanin	Monthly measurements from 2012 to 2019	Field	Yang et al., 2020

NA = data not available

[a] Interpret results with care as responses of *Microcystis* were not discerned over the course of multiple days.

[b] Greatest recruitment observed on day 1 of the experiment. Sediments were collected from the field post-bloom and stored in the dark at 4°C for a few days prior to experiment initiation.

[c] Cell densities increased more rapidly (1 day) at 16 °C and 25°C. By 12 days, cell densities were comparable at 4°C and 25°C.

3.4 Nutrients

Akinete germination was observed with no added sources of nitrogen and phosphorus in controlled laboratory environments (Table 6). Between 5% and 95% germination was measured in laboratory experiments that excluded sediment as a source of nutrients and utilized laboratory formulated media so the addition of nutrients could be limited (Huber 1985; Sutherland et al. 1985; Yamamoto 1976; Myers et al. 2010; Park et al. 2018). Concentrations of nitrate, ammonia, and phosphate were not analytically confirmed in laboratory experiments and multiple studies do not specify nominal concentrations of nutrients or germination percentages which limits the ability to interpret data. Two studies (Huber 1985; Myers et al. 2010) report percent germination of *Nodularia spumigena* akinetes following exposures to a series of nitrogen and phosphorus concentrations and refer to the treatments as nominal concentrations. These two studies provide data to aid in interpreting the potential impact of nutrient concentrations on akinete germination (Table 4). In Huber (1985), concentrations greater than 630 µg N/L as NH_4Cl inhibited germination (>45% decrease). Relatively low concentrations (27 µg P/L as K_2HPO_4) of phosphate and no known addition of nitrate resulted in the same germination percentages (75% and 95%, respectively) as the highest phosphate and nitrate concentrations evaluated. A dose-response relationship between akinete germination and nutrient concentrations (nitrate and phosphate) was measured in Myers et al. (2010). Nominal concentrations of 1,500 µg P/L to 2,500 µg P/L as K_2HPO_4 resulted in the greatest germination percentages (40%) (Myers et al. 2010). Nitrate amendments from 1,500 to 3,000 µg N/L as $NaNO_3$ resulted in the greatest percent germination (17% to 25%) (Myers et al. 2010).

As akinete germination was observed when there were no known additions of nutrients from sediment or aqueous sources in unrealistic laboratory conditions, nutrients are unlikely limiting the occurrence of akinete germination in the field. In aquatic systems, akinetes are located at the sediment-water interface where nutrient sources can be higher relative to the remaining water column. Additionally, akinetes may have the necessary compounds for germination contained internally. As previously mentioned in Chapter 2, akinetes contain greater concentrations of molecules that contain nitrogen (i.e., nucleic acid and cyanophycin) and phosphorus (i.e., nucleic acid) relative to vegetative cells. Although nutrients may not be required for germination, akinete-producing genera may germinate at higher percentages when nutrient concentrations are

within specific ranges and the sensitivity may be strain or site-specific (Huber 1985; Myers et al. 2010).

Studies demonstrating the responses of *Microcystis* overwintering cells to nutrient concentrations are limited (Table 7). In Stahl-Delbanco et al. (2003), nutrient cycling between the sediment and water were evaluated using enclosures inserted into sediments maintaining contact between sediment and water phases. Nutrient concentrations were measured following additions of nitrate and phosphate to the enclosures and the abundance of *Microcystis* was measured. *Microcystis* abundance in the unamended enclosures was less than $0.1x10^8$ cells/enclosure. *Microcystis* abundance was an order of magnitude (1.1 to 1.6 x 10^8 cells/enclosure) greater in the enclosures with 498 µg N/L as nitrate and ammonium and 134 µg P/L as total phosphorus relative to all other enclosures. In a different study, lake sediments from two locations ("near-shore" and "mid-lake") were placed in experimental chambers with water that was stripped of dissolved ions and nutrients (milli-Q) prior to amendments with ammonium chloride (Borges et al. 2016). Relatively low cell densities were measured (< 2,000 cells/mL) for the unamended controls and all amendments using one of the sediment samples ("near-shore"). For the other sediment sample ("mid-lake"), cell densities doubled at 100 µg N/L to 500 µg N/L as NH_4Cl relative to the unamended control and 1,000 µg N/L to 5,000 µg N/L as NH_4Cl. These results suggest that *Microcystis* cell densities increase as concentrations of nitrate, ammonia, and phosphate increase. It is likely that the perceived sensitivity of *Microcystis* to nutrient concentrations can range from site to site. However, measurements of nitrate, ammonia, and phosphate at the sediment-water interface may provide some predictive capability for overwintering *Microcystis* growth rates in water bodies.

Table 6. Influence of nutrients (nitrate, ammonia, and phosphate) on germination of akinetes.

Genera	Experimental conditions		Nutrient addition	Response/ Percent germination	Duration (days)	Reference
	Sediment	Overlying water				
Laboratory/bench-scale Observation						
Anabaena	none/ water only	Detmer medium (Wantanabe 1960)	No added nitrate addition*	24-26% germination. No impact on germination	6, germination percentage began to plateau at 1 day	Yamamoto 1976
Anabaena	none/ water only	Allen and Arnon's (nitrogen free) medium (Allen and Arnon 1955)	Phosphate and nitrate addition*	96%	5, maximum germination observed after 3 days	Rai and Pandey 1981
			Phosphate addition*	40%		
			Nitrate addition*	30-40%		
Nodularia	none/ water only	BG11-2 (nitrogen free) medium (modified from Hughes et al., 1958)	No added phosphate and 27 to 6,937 µg P/L as K_2HPO_4	0% and 70% germination occurred with no added phosphate for two separate replicates. With phosphate additions > 27 µg P/L as K_2HPO_4, 75% germination occurred	5	Huber 1985
			No added ammonium to 2,002 µg N/L as NH_4Cl	60% to 85% germination occurred with no added ammonium to <630 µg N/L as NH_4Cl. With ammonium additions > 630 µg N/L as NH_4Cl, 0 - 20% germination.		
			No added nitrate to 9,996 µg N/L as $NaNO_3$	90 – 95% germination at all concentrations		
Nostoc	none/ water only	BG-11o (nitrogen free) medium (Rippka et al., 1979)	No phosphate or nitrate addition	Germination observed*	2	Sutherland et al., 1985

Genera	Experimental conditions		Nutrient addition	Response/ Percent germination	Duration (days)	Reference
	Sediment	Overlying water				
Nodularia	none/ water only	MLA medium (modified phosphorus free Bolch & Blackburn 1996)	No added N to 3,000 µg N /L as $NaNO_3$	Observed increasing germination with increase in N. Maximum germination observed at 1,500 and 3,000 µg N /L as $NaNO_3$	8, germination observed at 3 days.	Myers et al., 2010
			No added P to 2,500 µg P /L as K_2HPO_4	Observed increasing germination with increase in P. Maximum germination observed at 1,500 and 2,500 µg P /L as K_2HPO_4		
Dolichospermum	none/ water only	CB medium (Wantanabe and Kasai 1985)	No added phosphate or nitrate	<5%	3	Park et al., 2018
			Phosphate and nitrate addition*	10%		
			Phosphate addition*	10%		
			Nitrate addition*	20%		
Dolichospermum	sediment from lake	CB medium (Wantanabe and Kasai 1985)	No added phosphate or nitrate	5%	3	Park et al., 2018
			Phosphate and nitrate addition*	80%		
			Phosphate addition*	85%		
			Nitrate addition*	70%		
Field Observation						
Dolichospermum	*in situ*	*in situ*	Non-detect to 2,800 µg/L nitrite-nitrate	Germination observed*	Not applicable	Kim et al., 2005

* Concentration or percent not specified.

[a] Nutrients and other environmental factors were altered within the same treatments. In addition to nutrients, salinity and light were altered within the same treatments in Myers et al., 2010. In Kim et al., 2005, this experiment was conducted *in situ* and environmental factors (e.g., temperature, light) in addition to nutrients were not controlled.

Table 7. Influence of nutrients (nitrate, ammonia, and phosphate) on *Microcystis* overwintering cells.

Experimental conditions		Response measurement	Nutrient concentration[a] or addition[b]	Magnitude of response	Duration (days)	Reference
Sediment	Overlying water					
Laboratory grow out						
Lake sediment collected from "mid-lake" and "near shore"	Milli-Q water	Cell density	No addition	2,000 cells/mL for "mid-lake" 1,500 for "near-shore" samples at day 9	12	Borges et al., 2016
			100 µg N/L as NH_4Cl	4,500 cells/mL for "mid-lake" and 1,500 cells/mL "near-shore" at day 9		
			200 µg N/L as NH_4Cl	4,300 cells/mL for "mid-lake" and 1,500 cells mL for "near-shore" at day 9		
			500 µg N/L as NH_4Cl	4,000 cells/mL for "mid-lake" and 1,500 cells/mL for "near-shore at day 9		
			1,000 µg N/L as NH_4Cl	2,100 cells/mL for "mid-lake" and 2,000 cells/mL for "near-shore" at day 9		
			5,000 µg N/L as NH_4Cl	2,100 cells/mL for "mid-lake" and 2,000 cells/mL for "near-shore" at day 9		
In-lake Sediment Enclosures						
Lake sediments	lake water	Weighted average abundance (*Microcystis* number/enclosure). Abundance weighted by day with day 6 receiving the greatest weight	43 µg N/L nitrate and ammonium and 34 µg P/L total phosphorus	$<0.1x10^8$	6	Stahl-Delbanco et al., 2003[c]
			143 µg N/L nitrate and ammonium and 46 µg P/L total phosphorus	0.1 to 0.4 $x10^8$		
			498 µg N/L nitrate and ammonium and 134 µg P/L total phosphorus	1.1 to 1.6 $x10^8$		
			1900 µg N/L nitrate and ammonium and 225 µg P/L total phosphorus	0.1 to 0.3 $x10^8$		

[a] concentrations of N amended as $Ca(NO_3)_2$ and P amended as KH_2PO_4 measured in Stahl-Delbanco et al., 2003

[b] concentrations amended to experimental chambers using ammonium chloride in Borges et al., 2016

[c] Nutrients and other environmental conditions likely altered within the same treatment. This experiment was conducted *in situ* and environmental factors (e.g., temperature and light) in addition to nutrients were not controlled.

3.5 Dissolved oxygen

Data relevant to dissolved oxygen concentrations as drivers for akinete germination and *Microcystis* bloom generation are limited. Kim et al. (2005) measured oxygen concentrations and akinete germination in the sediment of a reservoir. Germination of akinetes was discerned by observing morphological changes of the akinete envelope. Akinetes were considered to have "germinated" if the apical lid of the akinete envelope had opened. Oxygen concentrations remained aerobic (7-17 mg O_2/L) throughout the sampling period and no correlations were observed between akinete germination and oxygen concentrations. Harris and Trimbee (1986) observed a correlation between a decrease in dissolved oxygen (oxygen concentration minimum of 0-1 mg O_2/L) concentrations followed ten days later by a shift in algal abundance to mostly *Microcystis*. Initial dissolved oxygen concentrations of 1-4 mg O_2/L resulted in the greatest rate of increase in colony densities ([colony/mL]/d) of *Microcystis* observed by Reynolds et al. (1981).

3.6 Littoral mixing and turnover

Multiple authors have hypothesized that mixing of the water column and sediment is an important driver for *Microcystis* bloom formation (Verspagen et al. 2004; Misson and Latour 2012; Borges et al. 2016; Feng et al. 2019). The basis for this hypothesis is that mixing from wind or thermocline turnover is likely required to transfer vegetative overwintering cells from the sediment phase to the planktonic phase where the bloom would be observed. The data to support these hypotheses are limited. However, there is evidence that internal cell buoyancy (e.g., shifts in aerotope cell size and glycogen concentration) alone is insufficient to result in the sustained resuspension of overwintering *Microcystis* cells that would be observed in a HAB.

3.7 Overwintering cell density

Multiple authors have surveyed overwintering cells (i.e., akinetes and overwintering *Microcystis* colonies) in lake sediments (Table 8). Authors use different units to report overwintering cell densities on a per g, per cm^2, and per ml of sediment basis. Detected overwintering cell densities range from hundreds of cells per g or ml of sediment to tens of millions of cells per g sediment. Generally, densities are greater in the fall and winter relative to the spring and summer for akinetes (Kim et al. 2005) and

Microcystis overwintering colonies (Takamura et al. 1984; Brungerg and Blomqvist 2002). This is presumably because overwintering cells have been freshly deposited in the fall-winter prior to potential degradation of nonviable cells. Similarly, overwintering cell densities are greater in surficial sediments (0-2 cm from the sediment-water interface) likely because surficial sediments contain freshly deposited cells while sediments > 2 cm would contain older deposits (Takamura et al. 1984; Tsujmura and Okubo 2003; Kravchuk and Ivanova 2009).

In the late summer and fall, akinetes and vegetative cells would settle at (or near) the location of the HAB and once deposited to the sediments, would be impacted by the same forces that sort detritus by size classes (e.g., resuspension, settling, Stoke's law) in aquatic systems as time progresses. Akinete sizes can range from 5 to 50 μm with most akinetes less than 20 μm (e.g., *Anabaena* and *Dolichospermum*; Komárek and Zapomělová 2007). *Microcystis* colony sizes can range from <100 μm to 1,000 μm dependent on the individual cell size and total number of cells in the colony (Xiao et al. 2018). Higher densities of akinetes have been measured in sediments of smaller size classes (i.e., <4 μm to <63 μm for clay and silt) (Huber 1984; Kravchuk and Ivanova 2009) while little or no akinetes were identified in sandy sediments (> 63 μm for sand; Kravchuk and Ivanova 2009). Densities of akinetes were greater in areas of water bodies with lower water velocities such as dams (Cirés et al. 2013) and areas with dense submerged aquatic macrophyte growths that would slow water movement (Kravchuk and Ivanova 2009). Higher densities of akinetes (Cirés et al. 2013, Legrand et al. 2017) and *Microcystis* overwintering colonies (Reynolds 1981) have been discerned in deeper water columns.

Table 8. Reported cell densities and units of overwintering cells from the reviewed literature.

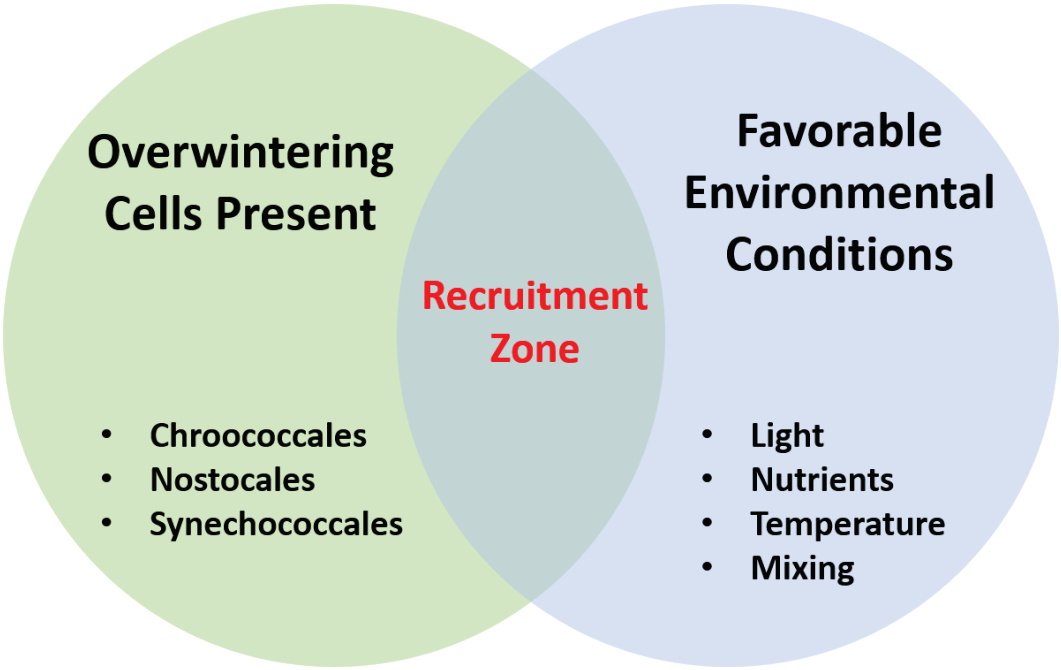
Overwintering cell	Minimum density (next highest detectable density)	Reference	Maximum density	Reference	Unit
Akinete	nd (150)	Legrand et al., 2017	36,000,000	Ramm et al., 2012	akinetes/g sediment
	nd (1,840)	Kravchuk and Ivanova 2009	61,000	Kravchuk and Ivanova 2009	akinetes/cm^2
	200	Cirés et al., 2013	186,900	Cirés et al., 2013	akinetes/ml sediment
Microcystis colony	nd (8,400)	Cirés et al., 2013	44,830,000	Cirés et al., 2013	cells/g sediment
	270,000	Brungerg and Blomqvist 2002	1,000,000	Brunberg and Blomqvist 2002	cells/cm^2

nd = non-detect

3.8 Recruitment zones

Recruitment zones are defined in this document as areas of sediment that, (1) contain viable overwintering cells, and (2) maintain favorable environmental conditions for periods of time necessary for akinete germination and overwintering cell growth (Figure 3). The recruitment zones would serve as areas with a viable source of potential HAB-producing cyanobacteria. Overwintering cells located outside of recruitment zones would presumably not have favorable environmental conditions to meaningfully contribute to planktonic blooms. Recruitment zones will be targeted as potential areas for preventative management during future research. Additionally, these areas can be targeted for sample collection to limit the amount of resources utilized in sampling the benthic environment if the goal of sampling is related to risk management.

Figure 3. Recruitment zones are areas that have viable overwintering cells and favorable environmental conditions for akinete germination and overwintering cell growth.



3.9 Summary

Water bodies that experience rapid recurring or seasonal and severe blooms may have overwintering cells that remain viable throughout the winter and proliferate during favorable environmental conditions in the spring. Genera within the orders Chroococcales, Nostocales, and Synechococcales are common HAB producers (i.e., are often identified in

samples from HABs and can produce toxins) and may survive as overwintering cells in the sediments. Genera of the orders Chroococcales and Synechococcales can overwinter as vegetative cells or colonies in the benthic environment. Alternatively, genera of the order Nostocales form akinetes or specialized overwintering cells. There are likely fundamental differences between these two overwintering cell types in terms of favorable conditions that would trigger cell growth of overwintering colonies or akinete germination.

Based on the literature reviewed, conditions necessary for akinete germination are as follows (Figure 4):

- Light: Photosynthetic photon flux densities of ≥ 0.5 μmol $m^{-2}s^{-1}$ or illuminance of 37 lx (assuming a conversion factor of 74), and
- Temperature: 22°C to 27 °C for maximum germination.

Based on the literature reviewed, conditions necessary for the growth of *Microcystis* overwintering colonies are as follows (Figure 5):

- Temperature: 15°C to 30°C,
- Nutrients: 100 μg N/L to 500 μg N/L as nitrate and ammonium and 134 μg P/L total phosphorus, and
- Mixing: resuspension of overwintering colonies.

Environmental conditions necessary for akinete germination and overwintering *Microcystis* growth are likely to occur in littoral zones and surface waters in the US.

Within waterbodies that experience HABs, reported densities of overwintering *Microcystis* colonies and akinetes in sediment can range five orders of magnitude. Based on this literature review, areas with the highest densities of overwintering cells were located at the surficial 0-2 cm of sediment. Studies have reported higher densities of akinetes in areas with relatively low flow rates such as dams and areas highly dominated by submerged aquatic macrophytes. Areas where vegetative cells may proliferate from overwintering cells are termed recruitment zones. These areas would have viable overwintering cells and meet the environmental conditions necessary for vegetative cell growth or akinete germination. This information can be used to inform field monitoring considerations for

identification of potential overwintering cell recruitment zones in HAB impacted waterbodies (Table 9).

Figure 4. Conceptual model of reported environmental conditions triggering the germination of akinetes.

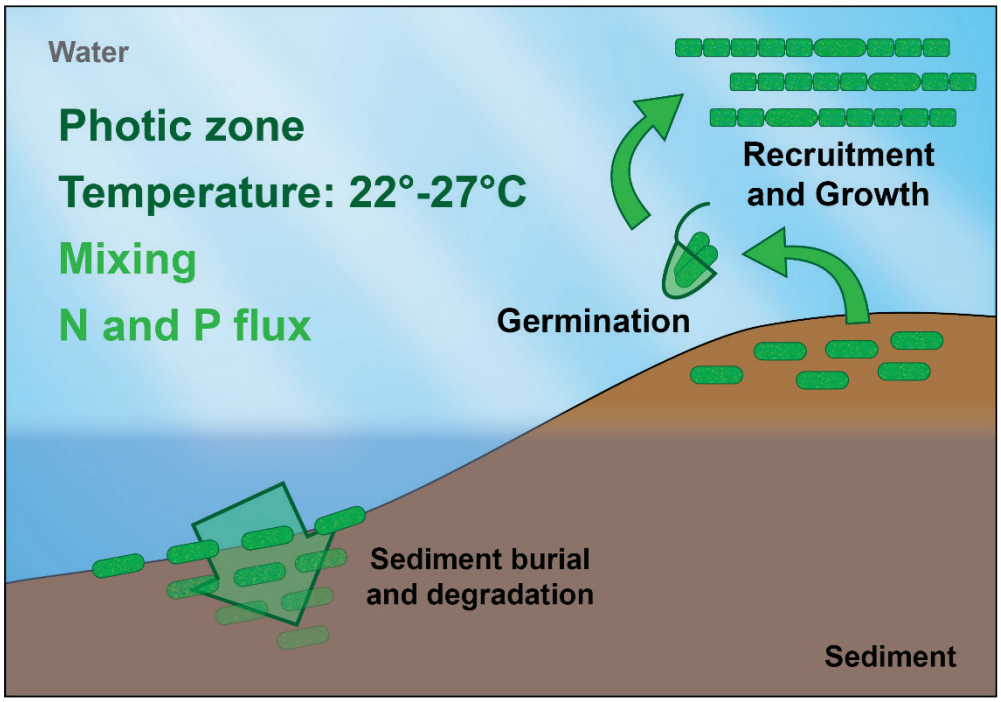


Figure 5. Conceptual model of reported environmental conditions triggering the growth of overwintering *Microcystis* colonies.

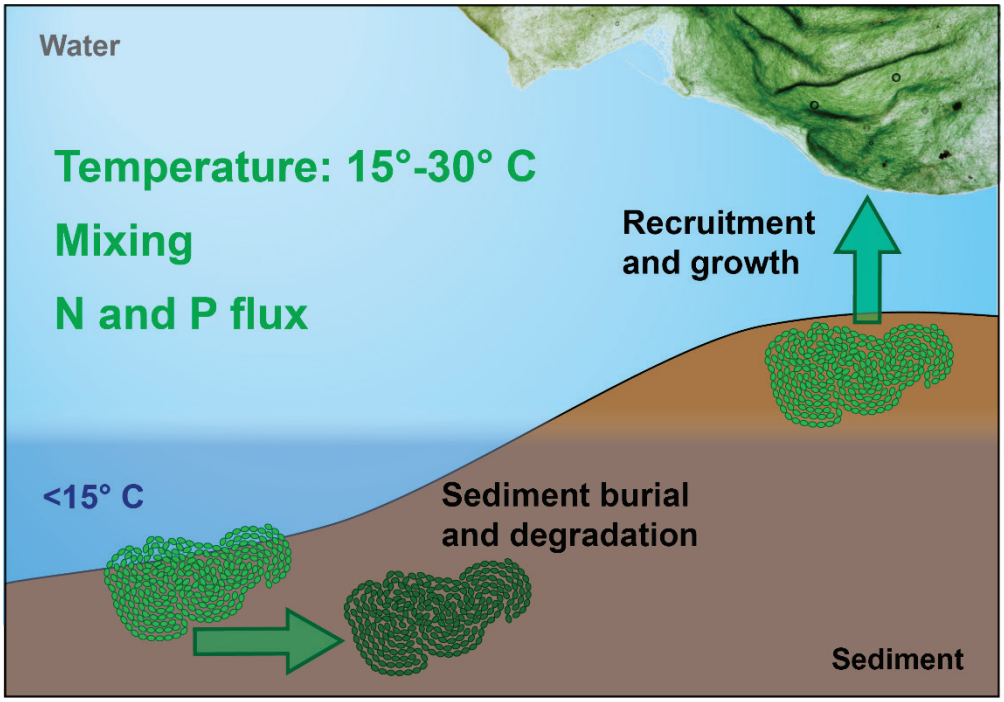


Table 9. Field monitoring considerations for identification of potential overwintering cell recruitment zones in HAB impacted waterbodies.

Target Sample Location	Target Data Needs	Target threshold criteria for akinete germination and rapid growth of overwintering cells
Overwintering Algal Cells in Sediments		
Sediment (0-2 cm at water-sediment interface)[a]	Identification and enumeration of overwintering cells	Presence of overwintering cells
Monitoring Environmental Conditions		
Overlying water at water-sediment interface	Nitrogen as nitrate-nitrite and ammonia	100 µg N/L to 500 µg N/L as sum of nitrate and ammonia for *Microcystis* overwintering cells
Overlying water at water-sediment interface	Phosphorus as soluble reactive phosphorus	134 µg P/L[b] for *Microcystis* overwintering cells
Overlying water at water-sediment interface	Light	≥ 0.5 µmol $m^{-2}s^{-1}$ or 37 lx
	Temperature	22°C to 27°C for akinetes
		15°C to 30°C for *Microcystis* overwintering cells
	Turbidity	Approximation for mixing with sediment phase

[a] For energetic systems, akinetes from deeper sediments may be suspended. Deeper sediments should be sampled.
[b] assumed that the majority of phosphorus was in a bioavailable form (soluble reactive phosphorus) as phosphorus was added as KH_2PO_4

4 Overwintering Cell Detection and Viability Metrics

Based on the considerations of the environmental tolerances and life cycles of HABs, which can overwinter in sediments, the following sections outline methods and approaches to consider for field monitoring.

4.1 Sampling methods

During sampling, care is needed to limit disturbance of the surficial sediment layer, so overwintering cells are not lost to resuspension. Sediment samples can be collected in shallow water depths with a hand core sampler such as a Lamotte soil sampling tube. At greater water depths, a dredge such as Wildco grab sampler, Ekman, or Ponar dredges can be used (Lind 1974; Calomeni et al. 2018). To limit the expense of transporting larger volumes of sediment, the surficial sediment (e.g., 0 to 2 cm) can be subsampled using a rubber spatula in the field. For energetic systems where deeper sediments may be resuspended leading to the germination of overwintering cells at depths >2 cm, a deeper surficial sediment sample may be needed. Surficial sediments containing overwintering cells can be stored on ice in a cooler to limit exposure to temperatures and light that may trigger germination or growth of overwintering cells.

4.2 Sediment-associated cyanobacteria identification and enumeration

The focus of this section is on identification and enumeration methods that can be conducted with tools available in many aquatic laboratories. It should be noted that there are numerous advancements in omics (e.g., ecogenomics, genomics, transcriptomics, proteomics, metabolomics) developed for cyanobacterial identification; however, the focus here was on widely available tools with relatively low technical and cost barrier. For enumeration of overwintering cells, a strategic review was conducted to identify techniques relatively easily implemented by laboratories that conduct enumeration of planktonic algal cells and could alter procedures for the enumeration of benthic overwintering cells. There are numerous inherent challenges associated with monitoring, sampling, and enumerating cyanobacteria in sediments, and standardized methodology is generally lacking (Wood et al. 2020). Segregation of benthic

overwintering cells from the surrounding sediment is one primary challenge to identification and enumeration; however, effective methods reported in the literature include sediment dilution, particle size separation, and density separation.

4.2.1 Dilution

A relatively simple method to limit sediment interference is the dilution of sediments. Researchers have utilized dilution factors of 1:9, 1:20, 1:50, and 1:100 g sediment to ml water (e.g., filtered site water or distilled water to remove planktonic algae) (Huber 1984; Tsujimura and Okubo 2003; Eilers et al. 2004; Rucker et al. 2009; Cirés et al. 2013; Bunting et al. 2016). After dilution, samples are homogenized by mixing or low energy sonication and a subsample is collected for enumeration. The dilution necessary for enumeration may be based on particle size of the associated sediment. Sandy sediments may require a lower dilution factor as the sand particles would rapidly settle before a subsample could be collected for placement on a microscope slide, thereby decreasing interference from sediment particles. Alternatively, clay sediments would remain suspended and transferred to the microscope slide requiring a greater dilution factor. Authors enumerated overwintering cells with Sedgwick-Rafter, Utermohl, and other plankton counting chambers placed on inverted microscopes or epifluorescent microscopes for enumeration. For quality assurance, authors reported counting between 100 and 400 cells which were equivalent to 10 to 120 fields of view depending on the cell densities of the samples.

4.2.2 Particle size separation

Knowledge of the approximate size of the target overwintering cell is necessary for separation of overwintering cells from sediment by particle size. Akinete sizes can range from 5 to 50 μm (*Anabaena* and *Dolichospermum*; Komárek and Zapomělová 2007). *Microcystis* colony sizes can range from <100 μm to 1,000 μm (Xiao et al. 2018). Rucker et al. (2009) was interested in discerning the cell density of *Microcystis* overwintering colonies and used mesh with a 40 μm pore size to remove sediment and detritus < 40 μm from samples while retaining overwintering colonies on the filter for enumeration along with larger sediment particles. Cirés et al. (2013) used two filters (41 μm and 0.2 μm pore size) of different sizes to separate sediments and associated overwintering cells into different size classes and enumerated

overwintering cells in each of the size classes. A potential issue that can occur with filtering is adding too much sediment to the filter. The excess sediment can impede the passage of particles that should pass through a filter with a specific pore size, therefore care is needed not to overburden and clog the filters.

4.2.3 Density separation

Density separation has been utilized by several authors (Vespagen et al. 2004; Cirés et al. 2013; Borges et al. 2016; Legrand et al. 2017; Table 10). Authors have used a polyvinylpyrrolidone coated silica sol, Percoll® (1.135 g/mL) (Vespagen et al. 2004; Cirés et al. 2013; Borges et al. 2016) and a silica sol Ludox TM-50® (density = 1.4 g/mL at 25 °C) (Legrand et al. 2017) for density separation of overwintering cells from sediment. Methods for separating overwintering cells from sediment vary based on mixture and centrifugation (Table 10) but can be modified for site-specific sediments. In short, Percoll® or Ludox TM-50® can be inverted with site water to generate a mixture with a density slightly greater than the overwintering cells. Densities of algae and cyanobacteria range from 1.076 to 1.100 g/mL (Cromar and Fallowfield 1992). A sediment subsample containing overwintering cells is then suspended in the mixture and centrifuged for 15 to 30 min. The supernatant containing the overwintering cells is removed for enumeration.

Table 10. Overview of reported methods for density separation of overwintering cells from sediments.

Density Separation Mixture	Amount of sediment and density separation mixture	Centrifugation	Additional cleanup	Algal Genera	Reference
7:3 Algal culture medium to Percoll®	Suspend 5 to 25 mL sediment in mixture	1,800 rpm for 15 minutes	yes	*Microcystis*	Vespagen et al., 2004
1:9 2.5M sucrose to Percoll®	3 g sediment to 30 mL mixture	20,000 g for 15 minutes	yes	*Aphanizomenon, Dolichospermum,* and *Microcystis*	Cirés et al., 2013
1:1 Algal culture medium to Percoll®	Suspend 6mL sediment in mixture	600 g for 15 minutes	yes	*Microcystis*	Borges et al., 2016
7:4 distilled water to Ludox®	3 ml sediment to 11 mL mixture	10,000 g for 30 min	no	*Dolichospermum*	Legrand et al., 2017

4.3 Grow out studies

Ultimately, the presence of overwintering cells does not invariably lead to the germination of akinetes or the growth of overwintering colonies. Additional lines of evidence can bolster data supporting the potential that overwintering cells are viable. Grow out studies utilize site collected

overwintering cells and simulate conditions understood to trigger akinete germination or overwintering cell growth. To conduct a grow out study, the experimental chamber is placed in the laboratory under favorable environmental conditions (e.g., light intensity and temperature) for several days. Growth is observed and planktonic cell densities are determined for 3 to 45 days (Livingstone and Jaworski 1980; Huber 1985; Park et al. 2018).

If the goal is to mimic or predict field conditions for the grow out study, sediment samples containing overwintering cells are placed in an experimental chamber (e.g., beaker, Erlenmeyer flask, test tube) and site water is added. The overlying site water can be filtered (0.45 µm pore size) to remove planktonic algal cells that may interfere with interpretation of the data from the growth of planktonic cells. These studies aim to ask the question, can overwintering cells germinate and grow under environmentally favorable conditions? Thus, using field collected sediment and water in grow out studies offer an additional line of evidence to indicate or predict that overwintering cells are viable in field conditions.

Researchers have also used algal culture media specifically formulated to promote the growth of cyanobacteria (e.g., BG-11) in lieu of filtered site water (Livingstone and Jaworski 1980; Huber 1985; Park et al. 2018). Grow out studies with culture media (e.g., BG-11) may be less predictive of the field relative to using site collected water. Culture media are artificially formulated to promote the growth of cyanobacteria and contain elevated concentrations of macro- and micronutrients that would likely not be present at the same concentrations in many water bodies. Grow out studies using culture media ask the question, can overwintering cells germinate in nutrient conditions in excess of cellular requirements? These studies offer an additional line of evidence to indicate or predict that overwintering cells are viable in field conditions if nutrient conditions increase in the study site in the future (i.e., non-point source nutrient input). If overwintering cells do not germinate in either site water or culture media, there would be two lines of evidence supporting that the overwintering cells will not germinate and grow and are thus non-viable.

4.4 Summary

Based on the reviewed literature, there are several approaches that aid in the monitoring and determination of overwintering cells in sediments (Table 11). Sediment sampling should be conducted to capture the 0-2 cm

layer at the sediment-water interface as this has the highest density of overwintering cells based on the reviewed literature. In energetic systems, sampling may need to capture sediment at depths greater than 2 cm if this layer has the potential to suspend (Table 12). A strategic review of available literature was conducted to identify overwintering cell enumeration techniques that could be implemented by laboratories that identify algae in water samples with relatively few alterations to procedures for identification of overwintering cells in sediment samples. Methods for decreasing interference from sediment include dilution and separation by particle size (sieves and filters) and density (centrifugation with polyvinyl pyrrolidone coated silica sol) (Table 13). Grow out studies utilizing site collected sediment and filtered site water (to remove planktonic algae) can be used to provide an additional line of evidence that the identified overwintering cells can germinate from an akinete or grow.

Table 11. Summary of approaches to answer questions related to overwintering cell enumeration and viability.

Question	Approach/ Methods
How many overwintering cells are present in the sediment?	Dilution and separation of overwintering cells followed by enumeration with microscopy
Are overwintering cells viable?	Grow out study with site collected sediment and water
Are overwintering cells viable when additional nutrients are added?	Grow out study with culture media

Table 12. Summary of primary factors sampling, identification, and enumeration methods of overwintering cells in sediments.

Sampling and enumeration methods	
Sampling	0-2 cm sediment at water-sediment interface with dredge or deeper for energetic systems
Identification and Enumeration	Sediment “cleanup” using dilution, sieving, or density separation followed by microscopy
Viability	Grow out studies with filtered site water or media

Table 13. Summary of primary factors influencing germination and growth of overwintering cells in sediments.

Factors triggering germination and growth	
Akinetes	≥ 0.5 µmol $m^{-2}s^{-1}$ or illuminance of 37 lx
	22°C to 27°C for maximum germination
Microcystis overwintering colonies	15°C to 30°C
	100 µg N/L to 500 µg N/L as nitrate and ammonium and 134 µg P/L total phosphorus
	Mixing of sediment/ water interface

5 Algaecide Treatment Efficacy of Benthic or Overwintering HABs

There is an extensive database demonstrating the efficacy of algaecides for the treatment of benthic cyanobacteria (Duke 2007; Bishop and Rodgers, 2011; Calomeni et al. 2015; Geer et al. 2017; Anderson et al. 2019); however, there are limited data focused on the treatment of overwintering cells. Some fundamental concepts that apply to algaecide treatments of benthic cyanobacteria will also apply to treatments of overwintering cells and will inform future research. For example, laboratory to field prediction of algaecide efficacy testing is well developed for benthic and plankton algae (Bishop and Rodgers 2011; Calomeni et al. 2015; Geer et al. 2017; Calomeni et al. 2018; Kinley-Baird et al. 2021). However, there are several factors that should be considered when implementing treatments using USEPA algaecides for the preventative management of overwintering cells associated with sediment. Factors that may impact the efficacy of USEPA registered algaecides for the treatment of overwintering cells are:

1. Algaecide activity in proximity to sediment (e.g., competing ligands)
2. Overwintering cell relative sensitivity to algaecide formulations
3. Timing of preventative treatments
4. Algaecide formulation, concentration, frequency and duration of exposure, and application method of treatment

Consideration of these factors during the planning stages of preventative treatments can inform treatment decisions and increase the likelihood of success.

5.1 Algaecide activity in proximity to sediment

USEPA registered algaecides include peroxide-based, copper-based (e.g., copper sulfate, chelated copper, acidified copper), and endothall-based algaecides. Peroxide-based algaecides may oxidize organic material in sediment resulting in a decrease in active ingredient available to oxidize algal cells (Geer et al. 2017). Copper-based algaecides may lose activity due to binding of the cupric ion (thought to be the active portion of the algaecide) with ligands in sediments including acid volatile sulfides and organic material (Calomeni et al. 2017). Endothall-based algaecides bind with sediment (Reinert and Rodgers 1984) and may be sequestered when applying treatments in proximity to sediments. Additionally, endothall can

degrade relatively quickly via microbial degradation and therefore contact time can vary considerably among different site conditions (Islam et al. 2018). Interactions with sediment need to be considered during the selection of specific algaecide formulations and concentrations to be utilized in field treatments. For example, concentrations of algaecide may need to be higher than what would typically be used in the water column for comparable cell densities due to the higher proportion of exposure modifying factors present near and in sediments. There are case studies supporting the efficacy of algaecide treatments for the management of vegetative algal cells when algae are near sediment (Section 5.5).

5.2 Overwintering cell relative sensitivity to algaecide formulations

One study was identified that evaluated the sensitivity of overwintering cells of *Microcystis* and *Anabaena* to different concentrations of hydrogen peroxide, H2O2 (Chen et al. 2016). Hydrogen peroxide is the active component in sodium carbonate peroxyhydrate-based algaecides. Sediment samples were collected in December from a site that experiences cyanobacterial blooms in the summer. Overwintering cells were initially screened from smaller sediment particles using a filter (63 µm pore size) and colonies were then removed from the filter surface with a pipette. Overwintering cells were exposed in filtered site water to a series of concentrations of H2O2 from 1 to 20 mg/L and cyanobacterial pigments (i.e., chlorophyll-α and phycocyanin) were measured for 72 hr following exposure. At 12 hr following exposure, pigment concentrations decreased from 35 µg/L to 22 µg/L and 75 µg/L to 40 µg/L for chlorophyll-α and phycocyanin, respectively at the two highest concentrations evaluated (5 mg/L and 20 mg H2O2/L). By 24 hr following exposure, phycocyanin concentrations (pigment contained by cyanobacteria only) was approximately half the concentration of the untreated control (80 µg/L) for all concentrations of H2O2 evaluated including the lowest concentration of 1 mg H2O2/L. In contrast, a study evaluating the relative sensitivities of vegetative *Microcystis aeruginosa* cells, determined that 1 mg H2O2/L was necessary to decrease chlorophyll-α concentrations by half. These data provide preliminary evidence that some overwintering cyanobacterial colonies are comparably sensitive to vegetative cells (Geer et al. 2016).

There are likely fundamental differences in sensitivities between akinetes and overwintering colonies of *Microcystis*. In theory, akinetes may be less sensitive to algaecides than vegetative cyanobacterial cells because of the

thick exterior envelop that may provide protection from exposures. To overcome potential insensitivities of akinetes, treatments may need to be initiated when germlings begin to emerge from the akinete envelop.

5.3 Timing of preventative treatments

The timing of preventative treatments will likely be critical for successful implementation of preventative treatments. In Jia et al. (2014), a preliminary experiment was conducted to discern the temperature that will trigger the growth of overwintering colonies of *Microcystis*. This temperature was determined to be 13 °C. Two preventative treatments using H2O2 followed by an application of rice straw were implemented in enclosures inserted into sediments at a site that experiences seasonal algal blooms. One treatment was applied in March and the other was applied when the water temperature reached approximately 13 °C. Chlorophyll-a, chlorophyll-b and phycocyanin were measured in the untreated enclosures and the treated enclosures from March to June. Two total increases in chlorophyll-a and phycocyanin concentrations occurred in April and June in the untreated enclosures. In April, chlorophyll-a and phycocyanin concentrations in the preventative treatment enclosures were approximately 30% less than the untreated enclosures (25 µg/L chlorophyll-a and 4 µg/L phycocyanin). In June, the pigment contained in cyanobacteria only, phycocyanin, was 60 % less than the untreated enclosures (5-6 µg/L phycocyanin). This study provides preliminary data that preventative treatments applied in the winter and as temperatures warm in the spring may decrease the severity of algal growths.

5.4 Algaecide formulation, concentration, duration of exposure, and method of treatment

Ultimately there are several site-specific factors that can impact preventative treatment success. Additionally, there are limited studies in the current peer-reviewed literature to provide data on the impact of sediment exposure modifying factors on preventative treatment efficacy, relative sensitivities of overwintering cells, and timing of treatments. To fill these data gaps, preliminary laboratory-scale experiments will be critical. As an analogous situation, preliminary laboratory-scale experiments have been utilized to inform selection of algaecide formulations, concentrations, and contact durations necessary for benthic and planktonic algae control (Bishop and Rodgers 2011; Calomeni et al. 2015; Geer et al. 2017; Calomeni et al. 2018; Kinley-Baird et al. 2021).

Preliminary experiments using sediment-containing overwintering cells collected from the site will be used to fill critical data gaps prior to pilot treatments. The critical data gaps that will be addressed include, (1) site-specific sensitivity of overwintering cells, and (2) the perceived sensitivity of overwintering cells in sediment.

5.5 Case studies

Based on the reviewed literature, there is evidence that algaecides have been successfully implemented in the field to manage issues posed by benthic cyanobacteria and sediment-associated algae (Table 14). In one field-scale effort to treat benthic mats of *Lyngbya wollei*, applications of different combinations of peroxide-based, copper-based, and endothall-based algaecides in three reservoirs led to a decrease in surface area of algal growths over 5 yr (Anderson et al. 2019). The acreage requiring treatment was decreased from approximately 170 to 80 acres, 125 to 15 acres, and 18 to 1 acre in Lay Lake, Jordan Lake, and Lake Mitchell, respectively (Anderson et al. 2019). In a separate case study, treatments of peroxide-based and copper-based algaecides in Hartwell Lake decreased concentrations of taste and odor compounds produced by an assemblage of cyanobacteria and diatoms (Huddleston et al. 2015). Concentrations of taste and odor compounds decreased from approximately 100 ng/L to 14 ng/L and 14 ng/L to 5 ng/L for 2-methylisoborneol and geosmin, respectively 12 days after treatment. These algaecide treatments decreased the number of customer complaints at the drinking water facility from off flavor caused by the taste and odor compounds produced by benthic algae. Additionally, the cost associated with removal of taste and odor compounds within the drinking water treatment facility was decreased. These studies provide evidence that algaecides can be effective when applied in proximity to sediments. Additionally, these studies demonstrate real-world applications that were effective for algal control.

Table 14. Summary of reported algaecide treatment efficacy of benthic or overwintering HABs.

Reported Observation	Alga	Algaecide Type/Compound Used	Result	Reference(s)
Potentially similar sensitivities of overwintering and planktonic *Microcystis* to a peroxide algaecide	*Microcystis*	Sodium carbonate peroxyhydrate[b]	96h EC_{50}: 0.9-1.0 mg/L as H_2O_2	Geer et al., 2016
	Microcystis[a]	Hydrogen peroxide	24h EC_{50}: 1.0 mg/L as H_2O_2	Chen et al., 2016
Preliminary demonstration of efficacy of peroxide for the preventative treatment of overwintering *Microcystis*	*Microcystis*[a]	Hydrogen peroxide; rice straw	Preventative treatments applied in winter and spring decreased HAB concentration measured as phycocyanin by 60% [c]	Jia et al., 2014
Preliminary laboratory efficacy studies can fill data gaps regarding perceived sensitivities of site collected overwintering cells	Assemblage including *Planktothrix, Anabaena, Oscillatoria*	Sodium carbonate peroxyhydrate	Effective algaecide concentration, formulation and contact duration predicted	Geer et al., 2017
	Lyngbya magnifica	Three copper-based algaecides; Sodium carbonate peroxyhydrate	Effective algaecide concentration and formulation predicted	Bishop and Rodgers 2011
Field-scale success treating benthic cyanobacteria associated with sediment	**Application using subsurface injection at approximately 0.3 - 0.6 m from sediment**			
	Lyngbya wollei	Three copper-based algaecides; Sodium carbonate peroxyhydrate; Liquid peroxide algaecide; Endothall	Approximately 80% decrease in treatment acreage because effective treatments decreased biomass of benthic HAB from year-to-year	Anderson et al., 2019
	Assemblage including *Planktothrix, Anabaena, Oscillatoria*	Copper-based algaecide and sodium carbonate peroxyhydrate	Approximately 75% decrease in taste and odor compounds produced by algae and decrease in customer complaints associated with off flavor in drinking water	Huddleston et al., 2015

NA = not applicable because study was conducted in a laboratory setting

[a] Study conducted with overwintering cells

[b] Sodium carbonate peroxyhydrate forms hydrogen peroxide in water

[c] Low phycocyanin concentrations measured overall. Untreated enclosures had 5-6 µg/L phycocyanin.

5.6 Data gaps

Data gaps that are apparent from this literature review and may impact preventative treatment efficacy include:

- Sensitivity of overwintering cells to algaecide exposures
- Relative sensitivities of akinetes, germlings and vegetative cells to algaecide exposures
- Perceived sensitivity of overwintering cells in sediment
- Sediment depths where there is sufficient algaecide activity to elicit a response
- Sediment depth in which overwintering cells can germinate and grow

Addressing these data gaps in future research will decrease the uncertainties associated with implementation of preventative treatment management approaches.

6 Summary

This study reviewed existing information on overwintering cyanobacterial cells to inform the feasibility of a preventative treatment approach. The review focused on (1) factors that may trigger germination and growth of overwintering cells in sediments, (2) sampling, identification, and enumeration methods for overwintering cells in sediment, and (3) identification of treatment approaches applicable to overwintering cells in sediments to prevent or minimize planktonic growth of HABs.

Overwintering cells include both akinetes (specialized cells of algae in the order Nostocales) and vegetative colonies associated with sediments (orders Chroococcales and Synechococcales). Factors that may trigger germination and growth of akinetes differ from factors that trigger the growth of overwintering colonies. There is consensus among the peer-reviewed literature that relatively consistent light (PPFDs of 0.5 to 100 μmol $m^{-2}s^{-1}$ or illuminance of 37 lx to 8,000 lx) is necessary for akinete germination. Temperatures from 22 °C to 27 °C resulted in the maximum germination percentages of the studies evaluated. For vegetative cells that overwinter in sediments, growth was triggered by temperatures ranging from 15°C to 30 °C. There were limited data on the impacts of nutrients on the growth of overwintering vegetative cells; however, two studies identified that nutrient concentrations from 100 μg N/L to 500 μg N/L as nitrate and ammonium and 134 μg P/L total phosphorus resulted in greater growth. It is hypothesized that mixing is another factor needed to transfer vegetative overwintering cells in the sediment to the planktonic phase where a bloom would be observed.

Based on the available literature, overwintering cell densities were greatest at 0-2 cm from the sediment-water interface. During sampling, dredges (e.g., Eckman, Ponar) can be used to collect initial sediment samples and the surficial layer can be skimmed using a rubber spatula. Methods for enumeration utilize microscopy for discerning overwintering cell densities. For enumeration, interference from sediment particles can be limited using an initial dilution step or separation of overwintering cells from sediment by particle size or density.

Multiple lines of evidence support that USEPA-registered algaecides can be utilized for the preventative treatment of overwintering cells. Data from two field studies demonstrate the scalability and efficacy of USEPA-

registered algaecides for the treatment of algae associated with sediments in water bodies. Preliminary data suggests that overwintering cells of *Microcystis* have similar perceived sensitivities to hydrogen peroxide (active ingredient in peroxide-based algaecides) as planktonic algae. Treatments conducted in enclosures placed in lake sediments, provided evidence that preventative management can decrease the severity of HABs. As is the case with planktonic algae, laboratory experiments can be used to discern relative sensitivities of overwintering cells to different algaecide formulations, durations of exposure, and concentrations necessary to achieve control. These experiments will be critical to refine candidate USEPA-registered algaecides for the preventative treatment of sediment-associated overwintering cells.

References

Adams, D. G., and P. S. Duggan. 1999. "Tansley Review No. 107. Heterocyst and akinete differentiation in cyanobacteria." *New Phytologist* 144(1): 3-33.

Anderson, W. T., J. N. Yerby, J. Carlee, W. M. Bishop, B. E. Willis, and C. T. Horton. 2019. "Controlling *Lyngbya wollei* in three Alabama, USA reservoirs: Summary of a long-term management program." *Applied Water Science* 9(8): 1-12.

Beaver, J. R., C. E. Tausz, K. C. Scotese, A. I. Pollard, and R. M. Mitchell. 2018. "Environmental factors influencing the quantitative distribution of microcystin and common potentially toxigenic cyanobacteria in US lakes and reservoirs." *Harmful Algae* 78, 118-128.

Bishop, W. M., and J. H. Rodgers. 2011. "Responses of *Lyngbya magnifica* Gardner to an algaecide exposure in the laboratory and field." *Ecotoxicology and Environmental Safety* 74(7): 1832-1838.

Borges, H., S. A. Wood, J. Puddick, E. Blaney, I. Hawes, D. R. Dietrich, and D. P. Hamilton. 2016. "Intracellular, environmental and biotic interactions influence recruitment of benthic *Microcystis* (Cyanophyceae) in a shallow eutrophic lake." *Journal of Plankton Research* 38(5): 1289-1301.

Brunberg, A. K., and P. Blomqvist. 2002. "Benthic overwintering of *Microcystis* colonies under different environmental conditions." *Journal of Plankton Research* 24(11): 1247-1252.

Bunting, L., P. R. Leavitt, G. L. Simpson, B. Wissel, K. R. Laird, B. F. Cumming, A. St. Amand, and D. R. Engstrom. 2016. "Increased variability and sudden ecosystem state change in Lake Winnipeg, Canada, caused by 20th century agriculture." *Limnology and Oceanography* 61(6): 2090-2107.

Calomeni, A. J., K. J. Iwinski, C. M. Kinley, A. McQueen, and J. H. Rodgers Jr. 2015. "Responses of *Lyngbya wollei* to algaecide exposures and a risk characterization associated with their use." *Ecotoxicology and environmental safety* 116, 90-98.

Calomeni, A. J., K. J. Iwinski, A. D. McQueen, C. M. Kinley, M. Hendrikse, and J. H. Rodgers. 2017. "Characterization of copper algaecide (copper ethanolamine) dissipation rates following pulse exposures." *Water, Air, & Soil Pollution* 228(11): 444.

Calomeni, A. J., T. D. Geer, and J. Rodgers Jr. 2018. "Laboratory studies for prediction of responses of algae to algaecides in situ." *Journal of Aquatic Plant Management* 56s,59-66.

Chen, C., Z. Yang, F. Kong, M. Zhang, Y. Yu, and X. Shi. 2016. "Growth, physiochemical and antioxidant responses of overwintering benthic cyanobacteria to hydrogen peroxide." *Environmental Pollution* 219, 649-655.

Cirés, S., L. Wörmer, R. Agha, and A. Quesada. 2013. "Overwintering populations of *Anabaena*, *Aphanizomenon* and *Microcystis* as potential inocula for summer blooms." *Journal of Plankton Research* 35(6): 1254-1266.

Cole, R. A. 2006. *Freshwater aquatic nuisance species impacts and management costs and benefits at federal water resources projects*. ERDC/TN ANSRP-06-3. Vicksburg, MS: US Army Engineer Research and Development Center. https://erdc-library.erdc.dren.mil/jspui/handle/11681/5112.

Cromar, N. J., and H. J. Fallowfield. 1992. "Separation of components of the biomass from high rate algal ponds using Percoll R density gradient centrifugation." *Journal of Applied Phycology* 4(2): 157-163.

Duke, B. M. 2007. *Laboratory and field responses of target and non-target species to algaecide exposures*. Doctoral dissertation, Clemson University.

Eilers, J. M., J. Kann, J. Cornett, K. Moser, and A. S. Amand. 2004. "Paleolimnological evidence of change in a shallow, hypereutrophic lake: Upper Klamath Lake, Oregon, USA." *Hydrobiologia* 520(1): 7-18.

Fallon, R. D., and T. D. Brock. 1981. "Overwintering of *Microcystis* in lake Mendota." *Freshwater Biology* 11(3): 217-226.

Fay, P. 1988. "Viability of akinetes of the planktonic cyanobacterium *Anabaena circinalis*." In *Proceedings of the Royal society of London*. Series B. Biological Sciences, 234(1276): 283-301.

Feng, B., C. Wang, X. Wu, C. Tian, Y. Tian, and B. Xiao. 2019. "Involvement of microcystins, colony size and photosynthetic activity in the benthic recruitment of *Microcystis*." *Journal of Applied Phycology* 31: 223-233.

Geer, T. D., C. M. Kinley, K. J. Iwinski, A. J. Calomeni, and J. H. Rodgers Jr. 2016. "Comparative toxicity of sodium carbonate peroxyhydrate to freshwater organisms." *Ecotoxicology and Environmental Safety* 132: 202-211.

Geer, T. D., A. J. Calomeni, C. M. Kinley, K. J. Iwinski, and J. H. Rodgers. 2017. "Predicting in situ responses of taste-and odor-producing algae in a southeastern US reservoir to a sodium carbonate peroxyhydrate algaecide using a laboratory exposure-response model." *Water, Air, & Soil Pollution* 228(2): 53.

Graham, J. L., K. A. Loftin, A. C. Ziegler, and M. T. Meyer. 2008. *Guidelines for design and sampling for cyanobacterial toxin and taste-and-odor studies in lakes and reservoirs*. US Geological Survey.

Graham, J. L., N. M. Dubrovsky, G. M. Foster, L. R. King, K. A. Loftin, B. H. Rosen, and E. A. Stelzer. 2020. "Cyanotoxin occurrence in large rivers of the United States." *Inland Waters* 10(1): 109-117.

Hänel, A., T. Posch, S. J. Ribas, M. Aubé, D. Duriscoe, A. Jechow, Z. Kollath, D. E. Lolkema, C. Moore, N. Schmidt, H. Spoelstra, G. Wuchterl, and C. C. Kyba. (2018). "Measuring night sky brightness: methods and challenges." *Journal of Quantitative Spectroscopy and Radiative Transfer* 205, 278-290.

Herman, B. D., J. O. Eberly, C. M. Jung, and V. F. Medina. 2017. *Review and evaluation of reservoir management strategies for harmful algal blooms*. ERDC/EL TR-17-11. Vicksburg, MS: US Army Engineer Research and Development Center. https://usace.contentdm.oclc.org/digital/collection/p266001coll1/id/4288/rec/4.

Head, R. M., R. I. Jones, and A. E. Bailey-Watts. 1999. "An assessment of the influence of recruitment from the sediment on the development of planktonic populations of cyanobacteria in a temperate mesotrophic lake." *Freshwater Biology* 41(4): 759-769.

Heisler, J., P. M. Glibert, J. M. Burkholder, D. M. Anderson, W. Cochlan, W. C. Dennison, C. Gobler, Q. Dortch, C. Heil, E. Humphries, A. Lewitus, R. Magnien, H. Marshall, K. Sellner, D. Stockwell, D. Stoecker, and M. Suddleson. 2008. "Eutrophication and harmful algal blooms: a scientific consensus." *Harmful algae* 8(1): 3-13.

Huber, A. L. 1984. "Nodularia (Cyanobacteriaceae) akinetes in the sediments of the Peel-Harvey Estuary, Western Australia: potential inoculum source for *Nodularia* blooms." *Applied and Environmental Microbiology* 47(2): 234-238.

Huber, A.L. 1985. "Factors Affecting the Germination of Akinetes of *Nodularia spumigena* (Cyanobacteriaceae)." Applied and Environmental Microbiology 49(1):73-78.

Huddleston, M., J. H. Rodgers Jr, K. Wardlaw, T. Geer, A. Calomeni, S. Willett, J. Barrington, D. Melton, J. Chastain, M. Bowen, and M. Spacil. 2016. Adaptive water resource management for taste and odor control for the Anderson Regional Joint Water System.

Islam, M. S., T. D. Hunt, Z. Liu, K. L. Butler, and T. M. Dugdale. 2018. "Sediment Facilitates Microbial Degradation of the Herbicides Endothall Monoamine Salt and Endothall Dipotassium Salt in an Aquatic Environment." *International Journal of Environmental Research and Public Health 15*(10): 2255.

Jia, Y., Z. Yang, W. Su, D. Johnson, and F. Kong. 2014. "Controlling of cyanobacteria bloom during bottleneck stages of algal cycling in shallow Lake Taihu (China)." *Journal of Freshwater Ecology* 29(1): 129-140.

Kaplan-Levy, R. N., O. Hadas, M. L. Summers, J. Rücker, and A. Sukenik. 2010. Akinetes: dormant cells of cyanobacteria. Dormancy and resistance in harsh environments. In: Lubzens, E., Cerda, J., & Clark, M. (Eds.). (2010). Dormancy and resistance in harsh environments (Vol. 21). Springer Science & Business Media.

Kim, B. H., W. S. Lee, Y. O. Kim, H. O. Lee, and M. S. Han. 2005. "Relationship between akinete germination and vegetative population of *Anabaena flos-aquae* (Nostocales, Cyanobacteria) in Seokchon reservoir (Seoul, Korea)." *Archiv für Hydrobiologie* 163(1): 49-64.

Kinley-Baird, C., A. Calomeni, D. E. Berthold, F. W. Lefler, M. Barbosa, J. H. Rodgers, and H. D. Laughinghouse IV. 2021. "Laboratory-scale evaluation of algaecide effectiveness for control of microcystin-producing cyanobacteria from Lake Okeechobee, Florida (USA)." *Ecotoxicology and Environmental Safety* 207, 111233.

Kitchens, C. M., T. H. Johengen, and T. W. Davis. 2018. "Establishing spatial and temporal patterns in *Microcystis* sediment seed stock viability and their relationship to subsequent bloom development in Western Lake Erie." *PloS one* 13(11): e0206821.

Komárek, J. 2010. "Modern taxonomic revision of planktic nostocacean cyanobacteria: a short review of genera." *Hydrobiologia* 639(1): 231-243.

Komárek, J., and J. Mareš. 2012. "An update to modern taxonomy (2011) of freshwater planktic heterocytous cyanobacteria." *Hydrobiologia* 698(1): 327-351.

Komárek, J., and E. Zapomělová. 2007. "Planktic morphospecies of the cyanobacterial genus *Anabaena*= subg. *Dolichospermum*–1. part: coiled types." *Fottea* 7(1): 1-31.

Kravchuk, E. S., and E. A. Ivanova. 2009. Spatial and temporal dynamics of *Anabaena flos-aquae* akinetes in bottom sediments of a small Siberian reservoir.

Kromkamp, J. C., and L. R. Mur. 1984. "Buoyant density changes in the cyanobacterium *Microcystis aeruginosa* due to changes in the cellular carbohydrate content." *FEMS microbiology letters* 25(1): 105-109.

Latour, D., H. Giraudet, and J. L. Berthon. 2004. "Frequency of dividing cells and viability of *Microcystis aeruginosa* in sediment of a eutrophic reservoir." *Aquatic Microbial Ecology* 36(2): 117-122.

Legrand, B., A. H. Le Jeune, J. Colombet, A. Thouvenot, and D. Latour. 2017. "Akinetes may be representative of past nostocalean blooms: A case study of their benthic spatiotemporal distribution and potential for germination in a eutrophic lake." *Applied and Environmental Microbiology* 83(23): e01571-17.

Lind, O. T. 1974. Handbook of common methods in limnology. St. Louis, MO: The C. V. Mosby Company, pp. 153–179.

Linkov, I., F. K. Satterstrom, D. Loney, and J. A. Steevens. 2008. *The impact of harmful algal blooms on USACE operations*. ERDC/TN ANSRP-09-1. Vicksburg, MS: US Army Engineer Research and Development Center. http://el.erdc.usace.army.mil/ansrp/ansrp.html.

Livingstone, D., and G. H. M. Jaworski. 1980. "The viability of akinetes of blue-green algae recovered from the sediments of Rostherne Mere." *British Phycological Journal* 15(4): 357-364.

Miller, M. M., and N. J. Lang. 1968. "The fine structure of akinete formation and germination in *Cylindrospermum*." *Archiv für Mikrobiologie* 60(4): 303-313.

Misson, B., and D. Latour. 2012. "Influence of light, sediment mixing, temperature and duration of the benthic life phase on the benthic recruitment of *Microcystis*." *Journal of Plankton Research* 34(2): 113-119.

Myers, J. H., J. Beardall, G. Allinson, S. Salzman, and L. Gunthorpe. 2010. "Environmental influences on akinete germination and development in *Nodularia spumigena* (Cyanobacteriaceae), isolated from the Gippsland Lakes, Victoria, Australia." *Hydrobiologia* 649(1): 239-247.

Paerl, H. W., and V. J. Paul. 2012. "Climate change: links to global expansion of harmful cyanobacteria." *Water research* 46(5): 1349-1363.

Park, C. H., M. H. Park, K. H. Kim, J. H. Park, D. R. Kwon, N. Y. Kim, D. R. Kwon, N. Y. Kim, B. J. Lim, and S. J. Hwang. 2018. "Akinete germination chamber: An experimental device for cyanobacterial akinete germination and plankton emergence." *Harmful Algae* 72: 74-81.

Preston, T., W. D. P. Stewart, and C. S. Reynolds. 1980. "Bloom-forming cyanobacterium *Microcystis aeruginosa* overwinters on sediment surface." *Nature* 288(5789): 365-367.

Rai, A. K., and G. P. Pandey. 1981. "Influence of environmental stress on the germination of *Anabaena vaginicola* akinetes." *Annals of Botany* 48(3): 361-370.

Rajaniemi, P., P. Hrouzek, K. Kaštovska, R. Willame, A. Rantala, L. Hoffmann, J. Komarek, and K. Sivonen. 2005. "Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon, Trichormus* and *Nostoc* (Nostocales, Cyanobacteria)." *International Journal of Systematic and Evolutionary Microbiology* 55(1): 11-26.

Ramm, J., A. Lupu, O. Hadas, A. Ballot, J. Rücker, C. Wiedner, and A. Sukenik. 2012. "A CARD-FISH protocol for the identification and enumeration of cyanobacterial akinetes in lake sediments." *FEMS Microbiology Ecology* 82(1): 23-36.

Ramm, J., J. Rücker, M. Knie, and B. Nixdorf. 2017. "Lost in the dark: estimation of the akinete pool for the recruitment of Nostocales populations (cyanobacteria) in a temperate deep lake." *Journal of Plankton Research* 39(3): 392-403.

Reinert, K. H., and J. H. Rodgers. 1984. "Influence of sediment types on the sorption of endothall." *Bulletin of environmental contamination and toxicology* 32(1): 557-564.

Reynolds, C. S., and A. E. Walsby. 1975. "Water-blooms." *Biological reviews* 50(4): 437-481.

Reynolds, C. S., G. H. M. Jaworski, H. A. Cmiech, and G. F. Leedale. 1981. "On the annual cycle of the blue-green alga *Microcystis aeruginosa* Kütz. emend. Elenkin. Philosophical Transactions of the Royal Society of London." *B, Biological Sciences* 293(1068): 419-477.

Rosen, B. H., and A. S. Amand. 2015. *Field and laboratory guide to freshwater cyanobacteria harmful algal blooms for Native American and Alaska Native Communities* (No. 2015-1164). US Geological Survey.

Ruecker, J., E. I. Tingwey, C. Wiedner, C. M. Anu, and B. Nixdorf. 2009. "Impact of the inoculum size on the population of Nostocales cyanobacteria in a temperate lake." *Journal of Plankton Research* 31(10): 1151-1159.

Sedmak, B., and G. Kosi. 1997. "Microcystins in Slovene freshwaters (Central Europe)—first report." *Natural toxins* 5(2): 64-73.

Ståhl-Delbanco, A., L. A. Hansson, and M. Gyllström. 2003. "Recruitment of resting stages may induce blooms of *Microcystis* at low N: P ratios." *Journal of Plankton Research* 25(9): 1099-1106.

Sukenik, A., R. N. Kaplan-Levy, J. M. Welch, and A. F. Post. 2012. "Massive multiplication of genome and ribosomes in dormant cells (akinetes) of *Aphanizomenon ovalisporum* (Cyanobacteria)." *The ISME Journal* 6(3): 670-679.

Sutherland, J. M., J. Reaston, W. D. Stewart, and M. Herdman. 1985. "Akinetes of the cyanobacterium Nostoc PCC 7524: macromolecular and biochemical changes during synchronous germination." *Journal of General Microbiology* 131(11): 2855-2863.

Takamura, N., M. Yasuno, and K. Sugahara. 1984. "Overwintering of *Microcystis aeruginosa* Kütz. in a shallow lake." *Journal of Plankton Research* 6(6): 1019-1029.

Trimbee, A. M., and G. P. Harris. 1984. "Phytoplankton population dynamics of a small reservoir: use of sedimentation traps to quantify the loss of diatoms and recruitment of summer bloom-forming blue-green algae." *Journal of Plankton Research* 6(5): 897-918.

Tsujimura, S., H. Tsukada, H. Nakahara, T. Nakajima, and M. Nishino. 2000. "Seasonal variations of *Microcystis* populations in sediments of Lake Biwa, Japan." *Hydrobiologia* 434(1): 183-192.

Tsujimura, S., and T. Okubo. 2003. "Development of *Anabaena* blooms in a small reservoir with dense sediment akinete population, with special reference to temperature and irradiance." *Journal of Plankton Research* 25(9): 1059-1067.

United States Environmental Protection Agency (USEPA). 2002. Method 1003.0: Green alga, *Selenastrum capricornutum*, growth test: Chronic toxicity. Excerpt from: Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA-821-R-02-013.

USEPA. 2010. National Aquatic Resource Surveys. National Lakes Assessment 2007 (NLA 2007 Profile-Data 20091008.csv). Available from USEPA website: http://www.epa.gov/national-aquatic-resource-surveys/data-national-aquatic-resource-surveys. Date accessed: 2021-07-16.

United States Geological Survey (USGS). 2020. *WaterQuality watch-continuous real-time water quality of surface water*. Available from USGS website: https://waterwatch.usgs.gov/wqwatch/animate?period=year&pcode=00010. Date accessed: 2021-07-16.

Vasconcelos, V., J. Morais, and M. Vale. 2011. "Microcystins and cyanobacteria trends in a 14 year monitoring of a temperate eutrophic reservoir (Aguieira, Portugal)." *Journal of Environmental Monitoring* 13(3): 668-672.

Verspagen, J. M., E. O. Snelder, P. M. Visser, J. Huisman, L. R. Mur, and B. W. Ibelings. 2004. "Recruitment of Benthic *Microcystis* (cyanophyceae) to the Water Column: Internal Buoyancy Changes or Resuspension? 1." *Journal of Phycology* 40(2): 260-270.

Wacklin, P., L. Hoffmann, and J. Komárek. 2009. "Nomenclatural validation of the genetically revised cyanobacterial genus *Dolichospermum* (Ralfs ex Bornet et Flahault) comb. nova." *Fottea* 9(1): 59-64.

Wood, S. A., L. T. Kelly, K. Bouma-Gregson, J. F. Humbert, H. D. Laughinghouse IV, J. Lazorchak, T. G. McAllister, A. McQueen, K. Pokrzywinski, J. Puddick, C. Quiblier, L. A. Reitz, K. G. Ryan, Y. Vadeboncoeur, A. Zastepa, and T. W. Davis. 2020. "Toxic benthic freshwater cyanobacterial proliferations: challenges and solutions for enhancing knowledge and improving monitoring and mitigation." *Freshwater Biology*, 65(10): 1824-1842.

Xiao, M., M. Li, and C. S. Reynolds. 2018. "Colony formation in the cyanobacterium *Microcystis*." *Biological Reviews* 93(3): 1399-1420.

Yamamoto, Y. 1976. "Effect of some physical and chemical factors on the germination of akinetes of *Anabaena cylindrica*." *The Journal of General and Applied Microbiology* 22(6): 311-323.

Yang, Z., M. Zhang, Y. Yu, and X. Shi. 2020. "Temperature triggers the annual cycle of *Microcystis*, comparable results from the laboratory and a large shallow lake." *Chemosphere* 260, 127543.

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14. ABSTRACT

Freshwaters can experience growths of toxin-producing cyanobacteria or harmful algal blooms (HABs). HAB-producing cyanobacteria can develop akinetes, which are thick-enveloped quiescent cells akin to seeds in vascular plants or quiescent colonies that overwinter in sediment. Overwintering cells produce viable "seed beds" for HAB resurgences and preventative treatments may diminish HAB intensity. The purpose of this literature review was to identify (1) environmental factors triggering germination and growth of overwintering cells, (2) sampling, identification, and enumeration methods, and (3) feasibility of preventative algaecide treatments. Conditions triggering akinete germination (light $\geq$0.5 µmol $m^{-2}s^{-1}$, temperature 22-27°C) differ from conditions triggering overwintering *Microcystis* growth (temperature 15-30°C, nutrients, mixing). Corers or dredges are used to collect surficial (0-2 cm) sediment layers containing overwintering cells. Identification and enumeration via microscopy are aided by dilution, sieving, or density separation of sediment. Grow-out studies simulate environmental conditions triggering cell growth and provide evidence of overwintering cell viability. Lines of evidence supporting algaecide efficacy for preventative treatments include (1) field studies demonstrating scalability and efficacy of algaecides against benthic algae, (2) data suggesting similar sensitivities of overwintering and planktonic *Microcystis* cells to a peroxide algaecide, and (3) a mesocosm study demonstrating a decrease in HAB severity following preventative treatments. This review informs data needs, monitoring techniques, and potential efficacy of algaecides for preventative treatments of overwintering cells.

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