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Spatial and temporal variation of suspect screening derived cyanobacterial secondary metabolite mixtures during harmful algal bloom events in shallow agroecosystem lakes

Stanley W. Kohls, [ab] Lyndy Holdt,[cd] Nervana Metwali,[c] Corey D. Markfort,[ab] Peter S. Thorne[c] and Gregory H. LeFevre *[ab]

Freshwater harmful algal blooms (HABs) pose significant health risks to communities through drinking water sources and contact recreation. Cyanobacteria that drive HABs produce a complex array of secondary metabolites, notably cyanotoxins. The spatiotemporal variability of cyanometabolite mixtures during HAB events remains poorly understood, particularly in agroecosystems, where heavy nutrient loading can alter normal lake dynamics. Therefore, we applied high-resolution mass spectrometry-based suspect screening to characterize cyanometabolite mixture dynamics during active HAB events in two Eastern Iowa lakes as test sites, using time controlled sampling at one lake and locationally controlled sampling at the other. Using the CyanoMetDB to enable complex mixture comparisons alongside ELISA-based microcystin (MC) quantification, we tentatively identified 38 unique cyanometabolites, revealing substantial chemical diversity beyond traditional monitoring targets. Spatial analysis at one of the lakes exhibited dramatic variability across a 104-meter beach, where MC concentrations ranged from 0.65 μg L^{-1} (below advisory limits) to 36.86 μg L^{-1} (4.6-fold greater than the USEPA health advisory threshold), with metabolite mixtures exhibiting low similarity ($\cos(\theta) = 0.38$) for those same sites. Temporal monitoring at the other test lake demonstrated rapid temporal changes, with MC concentrations dropping from 19.98 to 8.64 μg L^{-1} within one hour; however, the cyanometabolite mixtures over the same time were similar ($\cos(\theta) = 0.97$). Collectively, these findings demonstrate that HAB chemical composition and toxicity fluctuate substantially over short distances and time scales. This first HAB metabolite suspect screening in agroecosystem lakes thus provides a useful initial framework for characterizing overlooked cyanometabolite diversity and tracking chemical dynamics, advancing efforts to better understand, monitor, and mitigate HAB-related exposure.

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Environmental significance

Freshwater harmful algal blooms (HABs) are increasingly observed worldwide, driven in part by agricultural nutrient runoff, which can leave freshwater systems susceptible to bloom formation. These events generate a myriad of cyanobacterial secondary metabolites (CSMs), including cyanotoxins, generating exposure risk for humans and ecosystems. This study characterizes the occurrence and spatiotemporal dynamics of complex CSM mixtures in two agroecosystem lakes undergoing active HABs. By extending the analytical scope beyond commonly monitored microcystins through a high-resolution mass spectrometry suspect-screening approach, we identified chemical patterns that were undetected using conventional methods. The findings demonstrate how comprehensive suspect screening can yield previously inaccessible insights into the fine-scale chemical mixture dynamics underlying HAB development and progression.

Introduction

Freshwater harmful algal blooms (HABs) are a global water-quality problem that pose important and emerging human and environmental health risks. HABs are increasing in frequency and severity worldwide, driven at least in part by nutrient pollution and rising global temperatures.[1,2] Caused by cyanobacteria, HABs can produce a variety of cyanotoxins, including potent neurotoxins (anatoxin-a, saxitoxin) and hepatotoxins (microcystins, nodularins), posing significant risks to

[a]Department of Civil and Environmental Engineering, University of Iowa, 4105 Seamans Center, Iowa, 52242, USA. E-mail: gregory-lefevre@uiowa.edu; Tel: +319 335 5655

[b]IIHR—Hydroscience and Engineering, University of Iowa, 100 C. Maxwell Stanley Hydraulics Laboratory, Iowa, 52242, USA

[c]Department of Occupational and Environmental Health, University of Iowa, Iowa City, Iowa 52242, USA

[d]Friends of Lakeside Lab, 1838 Highway 86, Milford, IA 51351, USA

communities that rely on surface waters for drinking water and recreation (*e.g.*, swimming).[3,4] For example, during the 2014 Toledo (Ohio USA) water crisis, more than 400 000 residents lost access to tap water due to unsafe levels of HAB derived microcystins.[5] Recreational exposure to HABs and associated cyanotoxins can occur through accidental ingestion of water, inhalation of aerosols, or dermal contact.[6] Although HAB dynamics are complex, excessive nutrient pollution (*i.e.*, nitrogen and phosphorus) is a known driver of HAB occurrence,[7] with examples of nutrient control strategies being able to decrease the propensity for HABs.[8–10] Agricultural land use is strongly associated with excess nutrient loading, making agroecosystems particularly vulnerable to HAB events.[11,12] In Iowa, where ~85% of land is dedicated to agriculture, HABs are recurring problems in lakes and reservoirs with at least six and up to 50 unique microcystin derived swim advisories (microcystin concentrations >8 μg L^{-1}) issued by the Iowa DNR annually between 2006 and 2022.[13,14] Effective management and risk abatement rely on accurate characterization of bloom chemistry and toxicity dynamics.

HABs produce a myriad of secondary metabolites, including cyanotoxins, resulting in spatiotemporally dynamic complex mixtures of cyanobacterial secondary metabolites (CSMs). CSM mixtures can include microcystins, anatoxin-a, saxitoxin congeners with unique toxicities, and other CSMs with often indeterminate toxicity.[15–17] Additionally, there are potential concerns regarding the production of non-proteinogenic amino acids (NRPs) by cyanobacteria (*i.e.*, BMAA) due to the potential connections to degenerative diseases.[18] Although microcystins, specifically microcystin-LR, are the most commonly reported cyanotoxins,[19] the complex mixture present could result in unexpected interactive exposure effects (*e.g.*, antagonistic or synergistic effects).[20,21] CSM mixtures of active HABs vary spatially and temporally because of factors controlling cyanobacterial activity and CSM fate, potentially including cyanobacterial population dynamics, environmental stressors, wind-driven lake mixing, and product degradation.[22–28]

Risk assessment typically relies on enzyme-linked immunosorbent assay (ELISA) tests, despite the known complexity of HABs. ELISA tests are effective for determining the concentration of bindable toxin in a sample and can be used in conjunction with a threshold value (*e.g.*, 8 μg per L microcystin in Iowa) to estimate risk, but ELISA cannot assess variability among individual microcystin congeners.[29] To quantify individual cyanotoxin congeners, targeted analytical techniques such as LC-MS/MS are the primary approach. Nevertheless, the need for a broad suite of expensive authentic standards increases analytical expense and limits the scope of possible analysis to only cyanotoxins with commercially available authentic standards.[20,30] Although both binding assays and targeted analytical approaches are critical for estimating risk from cyanotoxins during a HAB event, neither approach captures the dynamics of the complex CSM mixture, which is relevant to understanding HAB event mixture dynamics.

High-resolution mass spectrometry (HRMS) is an emerging analytical technique that enables screening of unknown chemical substances without analytical standards and thus facilitates examination of a broader chemical space. The CyanoMetDB, which aggregates 3085 identified CSMs from peer-reviewed papers as of 2023, is a complementary tool that enables analysis of the chemical fingerprint of HAB-relevant features within the observable chemical space using suspect-screening HRMS methods.[16,31] Although suspect screening cannot provide quantitative concentrations, this analytical approach allows for tentative identification of CSMs and their relative abundances, enabling more detailed analysis of the chemical spatiotemporal dynamics of complex mixtures within HAB systems. Since the publication of the CyanoMetDB, there has been a limited body of work applying the database towards CSM-focused suspect screening. For example, Wang *et al.* performed a comprehensive study of 859 samples collected over four years at Lake Greifensee, Switzerland.[32] The authors reported 46 unique cyanometabolites identified *via* the CyanoMetDB and targeted methods, demonstrating correlations between CSM mixture composition and cyanobacterial species.[32] Using 16 samples over the course of a year from Lake Karaoun, LB Zervou *et al.* measured 92 CSMs identified using targeted and CyanoMetDB enabled suspect screening.[17] In another study, Roy-Lachapelle *et al.* integrated targeted, suspect, and non-targeted analysis to identify 61 CSMs, including seven new transformation products and potential microcystins through predictive biotransformation tools coupled with their non-targeted analysis.[33] Additionally, Shang *et al.* observed 155 tentative CSMs from 22 sites in and around Shanghai, China.[34] Using an estimate to convert between CSM feature peak area and an estimated MC-LR_{eq} concentration, the researchers demonstrated a correlation between total CSM feature abundance and *T. platyurus* toxicity.[34] This limited but growing body of previous work proves the efficacy of applying suspect screening to CSM mixture characterization and compound identification. To date, however, the use of HRMS based techniques and the CyanoMetDB have yet to be applied to capture finer-scale spatiotemporal dynamics—with a particular critical need in agoecosystem lakes that experience heavy nutrient loading conditions such as in Iowa of the agriculturally-dominated Midwestern US.

The drivers of spatiotemporal dynamics of CSM mixtures, including cyanobacterial growth, bulk transport, and toxin production, remain poorly understood. Moreover, the expected spatiotemporal variability of the CSM mixtures creates the potential for co-exposure to several CSMs during contact recreation, but the extent of these co-exposures and the corresponding spatiotemporal variations in risk resulting from potential toxicant interactions (*e.g.*, additive, synergistic, antagonistic) are poorly understood. Knowledge of the spatial variability of CSMs is limited, with most research focusing on single-location sampling.[15,35,36] Additionally, temporal investigations of CSMs are typically limited to weekly or daily resolutions in many academic studies and most public health monitoring.[15,35,37] For example, the Iowa Department of Natural Resources (DNR) conducts a summer weekly beach monitoring program that reports microcystin concentrations from a composite sample collected at three points along the beach earlier in the week. Nevertheless, these spatially averaged and

time-delayed results may poorly represent the rapidly changing characteristics of HAB-impacted lake systems and may not represent the exposure conditions experienced by a water recreationalist.[15,27,38] There is a critical need to apply suspect screening approaches using the CyanoMetDB in an intensive agroecosystem region, such as Iowa, to characterize the spatiotemporal dynamics of an active HAB event to address these research gaps. Therefore, the objective of this study was to comprehensively quantify the spatiotemporal dynamics of CSM mixtures using suspect screening techniques in an agroecosystem region at test lakes experiencing active HAB events. With our focus on mixture analysis, the goal of this study was not to achieve high-confidence identifications or quantification, but rather to capture the broad dynamics of cyanobacteria-associated compounds. We hypothesized that when controlling for either location or time of samples during an active HAB event, significant variation in the CSM mixture would occur. Through this work, we were the first to apply the suspect screening approach to understand the dynamics of CSM mixtures in an intensive agroecosystem, providing an initial snapshot of spatial and temporal variability in CSM mixtures in two representative lakes with recurring blooms.

Methods and materials

Experimental design

Site description. Two test lake study locations, Lake MacBride and Lake Darling in eastern Iowa, USA, were selected as sampling sites (Fig. S1 and Table S1). The lakes, used for public recreation, are in watersheds with greater than 50% row crop agricultural land use, which is representative of lakes found in agroecosystems (detailed in Fig. S2). The two lakes were sampled during active HAB events (as indicated by microcystin detection in Iowa's weekly monitoring) to determine the impacts of sampling location and time, separately, on microcystin concentrations and the CSM mixture (conditions at time of sampling shown in Fig. S3).[14] Lake MacBride was sampled at two public access beaches on the lake, with one sample collected from each end of each beach on August 17th, 2022, at the same time (sampling duration: approximately 20–30 minutes) to allow spatially dependent comparisons of the sites. Lake Darling was sampled at one primary public access beach location over a 3.6-hour interval on August 7th, 2023, allowing investigation of the temporal evolution of CSM mixtures. The samples ($n = 7$) were collected from 11:20 to 15:00 ($t = 0$ hours to $t = 3.63$ hours), representing a typical time window for recreation. Although sampling occurred at only two lakes in this study, limiting the scope of fully generalizable conclusions, the sites provide a critical snapshot for agroecosystem lakes experiencing an active HAB event and thus provide useful insights on application of emerging analytical approaches to characterizing complex spatiotemporal mixture dynamics and thus represent the first study of this kind.

Sample collection. The sampling protocol was adapted from the Ohio EPA Method 546 (determination of total MCs in ambient water). Briefly, at each beach site, 200 mL surface water grab samples were taken. At each sampling location, the polyethylene sample dipper was washed in the water three times, to remove surface scum, at approximately 1 meter off the shore before taking about 200 mL of water to pour into a pre-labeled and sterilized 250 mL amber glass jar.[39] All samples were immediately stored on ice in a cooler, returned to the lab, and frozen at −20 °C until ELISA and suspect screening analyses (additional details in SI Section S2.1).

Analytical methods

ELISA. Samples were first subject to ELISA testing using Eurofins Abraxixs ELISA kits, following the manufacturer's instructions. ELISA testing was used for quantification of cyanotoxins over direct conger measurement *via* LC-MS/MS because the objective of this work focused on mixture dynamics, and ELISA tests can capture the concentration of the mixture of cyanotoxin congers potentially present. Additionally, ELISA is the commonly used approach for monitoring HABs from a public health perspective, and thus results provide context with publicly available data.[29] The kits used were the Microcystins-ADDA ELISA (product no. 520011, Microcystins-ADDA ELISA) and the Anatoxin-a Receptor Binding Assay (product no. 520050. Anatoxin-a ELISA) (detailed in SI Section S2.2). Anatoxin-a was only measured at the Lake MacBride site to check for consistency in the cyanotoxin concentration measurement (Table S8). It should be noted that following Ohio EPA Method 546 for the SCM product liberation (freeze-thaw), will result in neither the truly dissolved nor fully extracted concentrations of toxins being present in the measured samples.[39,40] Following ELISA, the remaining sample volume was returned to a −20 °C freezer and stored until ultra-high-performance liquid-chromatography coupled with high resolution mass spectrometry (UHPLC-HRMS) based suspect screening analysis.

UHPLC-HRMS. The remaining sample volume was vortexed to mix the sample to homogeneity, and a 2 mL aliquot was passed through a 0.22 μm PTFE syringe filter (13 mm diameter Luer lock from MDI Membrane Technologies [type SY13TF], used as packaged). No further enrichment or cleanup was conducted due to limited available sample volume (some samples were used in other studies and reduced available volume, and a consistent method was required for the samples), which also helped limit potential bias in the detectable chemical space.[31] Analytical blanks of Milli-Q water (18 MΩ cm) were also prepared. Filtered samples were analyzed at the UIowa HRMS Facility on a Thermo Q Exactive Orbitrap MS using a Poroshell 120 C18 HPLC column with an Eclipse Plus C18 guard column. The chromatography mobile phases consisted of water and acetonitrile with 0.1% formic acid at 0.4 mL min^{-1} (see Table S3 for gradient details). Injection volumes were 10 μL. All samples underwent two polarity-switching full scans and a positive- and negative-mode data-dependent MS2 scan (ddMS2) (See Tables S4 and S5 for detailed MS acquisition parameters). The mass scan range of the instrument was set to 100–1500 m/z to ensure capture of lower-molecular-weight compounds in the CyanoMetDB (118.13–11009.12 Da), while selecting the maximum m/z possible within the

instrumentation constraints, allowing capture of 96% of the identifiable CSM.[16] The Lake MacBride and Lake Darling samples were analyzed over two separate injection sequences (see Table S6 for the injection sequence). Analytical blanks were used to evaluate potential carryover between injection sets and to remove background.

Study reporting tool. The NTA Study Reporting Tool (SRT) was used in the preparation of this manuscript and is available as SI.[41]

Data processing and analysis

The data were maintained in .raw format and analyzed in Compound Discoverer (Thermo Scientific V3.3.3) using a suspect screening workflow modified from the preset *Environmental w Stats Unknown ID w Online and Local Database Searches* workflow (see Fig. S4 and Table S7 for details). Specifically, the CyanoMetDB V2 was included as a mass list for tentatively identifying features *via* a mass-list-matching suspect screening approach, although the database lacks associated MS2 data. Results were systematically filtered for peak quality and mass matching (see the complete list of filters applied in Fig. S5). When MS2 data were available, *in silico* fragmentation and spectra scoring were conducted using the structural information reported in the CyanoMetDB and Compound Discoverer's Fragment Ion Search (FISh) scoring (reported in Tables S14 and S18), respectively. Additionally, MS2 data were further investigated using MS2Query,[42] a reliable machine learning-based tool that generates analog and exact matches for MS2 spectra (see SI S3.2). MS2Query provides a secondary identification method for the CSMs; however, the GNPS library[43] on which the tool is based does not contain spectra for many of the CSMs in the CyanoMetDB.[16] Results from MS2Query can be found in Tables S10 and S11. Neither the FISh score nor the MS2Query results were used to further filter the dataset. The full mass-matching list was used for secondary analysis (*i.e.*, all confidence level 4 and above features) because critical information regarding the dynamics of CSM mixtures can be derived even at lower confidence levels, which is the primary goal of this study rather than generating a high-confidence CSM inventory.[44] A summary of the identification confidence levels, based on the Schymanski scale,[44] can be found in Table S9, with all subsequent references to specific features having their confidence level in [ ] after their name. Comparisons between tentatively identified features in each sample were based on relative abundance, as determined by the ratio between the feature's average peak area for an individual sample's full scan injections and the average response for the feature among all full scan injections for all samples. It must be recognized that matrix effects resulting from background sample composition may influence the feature peak area used for these comparisons, which may vary between sites and samples. To highlight temporal variability specifically, a time-zero-normalized log 10 response was used to quantify changes relative to the initial conditions. Hierarchical clustering analysis was conducted within Compound Discoverer using Euclidean distance and the complete linkage method. The relative abundance data for the tentatively identified feature list were used in R (version 4.4.1) to determine the cos(θ) similarity score between each sample to quantify the mixture's similarity with respect to all the tentatively identified features. The cos(θ) approach computes the angle between a higher-dimensional vector formed by a feature identifier (*i.e.*, the relative abundance) and a score vector, where one represents complete correlation, and zero indicates no correlation.

Results and discussion

High spatial variability of microcystins and cyanometabolite mixtures

The cyanotoxin concentrations and tentatively identified CSMs at Lake MacBride demonstrate spatial heterogeneity in mixture composition and exposure potential. ELISA-measured microcystin concentrations varied across the four sampling sites, ranging from 0.65 μg L^{-1} to 36.86 μg L^{-1}. Site South 2 exceeded Iowa's eight ppb advisory threshold by 4.6-fold, while the microcystin concentrations at the other three sites were less than 2.20 μg L^{-1} (Fig. 1b). Anatoxin-a concentrations (Table S8) were lower than the concentrations of microcystins (9.53 μg L^{-1} at South 2 and <1 μg L^{-1} at the other sites), but mirrored the spatial variation pattern, indicating consistent cyanobacterial activity.[45] These differences in ELISA-measured cyanotoxin concentrations reveal location-dependent exposure potential. Nevertheless, ELISA, as a receptor-binding assay, cannot identify or characterize the composition of CSMs present. We therefore employed an HRMS suspect-screening approach to analyze the observed complex CSM mixture.

Suspect screening enabled the tentative identification of thirty CSM features. Notably, only two were potentially microcystins (MC-RR [level 2(b)]and [D-Asp3]MC-HilR [level 3b]) and one a potential anatoxin-a (*cis*-carboxydihydroanatoxin-a [level 3a]). The remaining 27 features were other potential CSM peptides and non-peptides, with no saxitoxins or nodularins identified. A complete list of tentatively identified features with associated information from the CyanometDB, critical acquisition results, and scored MS2 spectra when available, are provided in Tables S12–S14. Additionally, it should be noted that these compounds were initially isolated from a diverse group of cyanobacterial species, including species from marine and freshwater systems from across the globe. Although the global breadth of the CyanoMetDB is a substantial asset due to the comprehensiveness of the database, this also presents a potential limitation for suspect screening approaches of a localized nature such as this study. Nevertheless, specific CSMs are known to be produced across different species and locations; for example, recent research has demonstrated high variability in the distribution of CSMs globally.[46] Moreover, specific CSM products have been isolated from multiple cyanobacterial species, such as anabaenopeptin F, which has been isolated from five different genus of cyanobacteria.[47] Microcystins have also been shown to be produced by marine species of cyanobacteria, though specific conger determination has yet to be performed.[48–50] The number of tentatively identified high-confidence features (as determined by *in silico* MS2 matches and MS2Query results) was low compared to

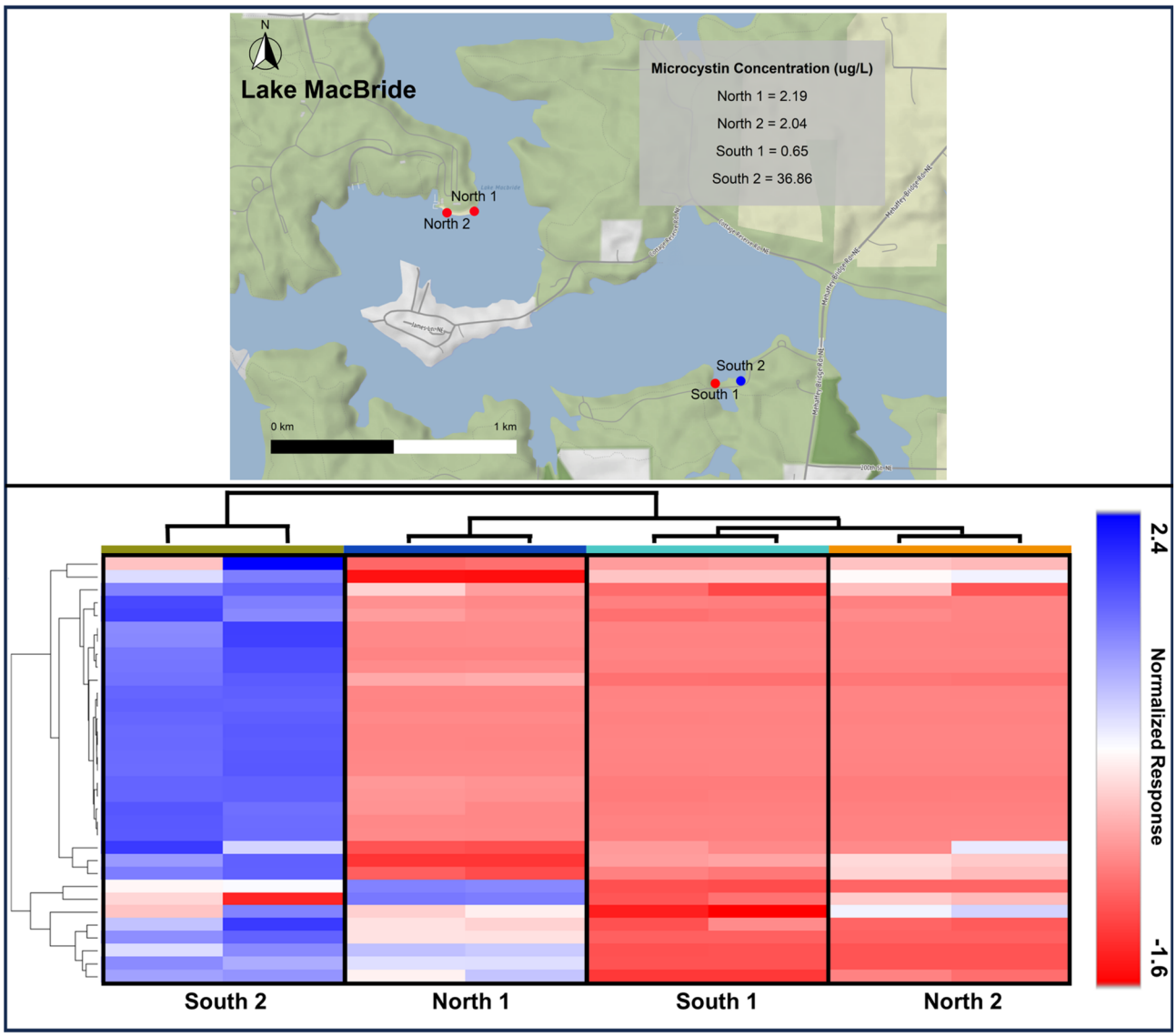

Fig. 1 The sampling site location map and associated ELISA-measured microcystin concentrations. The bottom panel shows the HCA plot of the 30 tentatively identified CSM features. Each row represents a single chemical feature, with cell color indicating its relative scaled response (blue: high; red: low). The dendrograms display the relatedness of both sites (columns) and features (rows), with groupings based on the complete linkage method using Euclidean distances and relative response values. As a binding assay, the ELISA-measured microcystin concentrations reflect total microcystin concentrations contributed from a mixture of congeners.

previous suspect screening work for CSM, potentially due to factors such as watershed land use, bloom species composition, or differences in analytical methods.[17,34,51] Nevertheless, the features, including mass matching only tentative identifications, expand the range of observed CSM beyond typically studied toxins, enabling critical insight into spatial variability across sampling locations.

Applying hierarchical cluster analysis (HCA) and cosine similarity ($\cos(\theta)$) analyses to the tentatively identified CSMs revealed distinctive mixture profiles across sites (Fig. 1). The dendrograms derived from the HCA indicate the similarity of sites (columns) and chemical features (rows) relatedness based on Euclidean distances derived from relative abundances of CSMs in the mixtures. The HCA identified Site South 2 as the most unique, with a Euclidean distance of 12.36 (unitless) from other sites, more than twice the maximum value observed between the remaining three sites. Cosine similarity mirrored the HCA findings, with Site South 2 exhibiting weak correlation to all other locations ($\cos(\theta)_{\text{MacBride}} < 0.46$). In contrast, Sites South 1 and North 2 were highly correlated ($\cos(\theta)_{\text{MacBride}} = 0.983$), despite a physical spatial separation of 1.4 km and being on opposite sides of the lake (see Table S15 for the full $\cos(\theta)_{\text{MacBride}}$ matrix). The high similarity may be a reflection of shared low relative abundance of CSM features at South 1 and North 2 rather than actual compositional similarity. Nevertheless, these results indicate a weak correlation between the sample location and the mixture composition, suggesting yet unexplained drivers of potential exposures.

Although spatial heterogeneity in algal bloom extent has been documented through remote sensing or pigment measurements, previous studies have not characterized the spatial variation of CSM mixtures.[23,27] The heterogeneity we observed may be expected, given that lakes are heterogeneous systems, with bloom extent, localized cyanobacterial activity, and beach-scale drainage basin variations likely driving the observed variation.[23,52] Nevertheless, the magnitude of this heterogeneity is notable. Most HAB monitoring programs sample individual locations or small numbers of sites, focusing on longer-term trends, which may mask the variability in potential exposure documented here.[17,34,53] For example, a recreationist at the South Site beach during our sampling period would have experienced a microcystin exposure 4.6-fold above the advisory threshold on the east side of the beach, but a potential exposure well below the threshold on the west side, only 103 m apart. In addition, a recreationist would have been exposed to a more pronounced complex mixture of CSM with an undetermined potential risk. Suspect screening thus provides a critical tool for revealing and characterizing HAB spatial dynamics from a CSM mixture perspective, enabling enhanced assessment of the spatially heterogeneous exposure imparted during active HAB events.

Temporal variation of microcystin and cyanometabolite mixtures

Samples were collected from one location over a 3.63-hour sampling period at Lake Darling's public access beach to document temporal HAB toxin and mixture variability. Over the sampling period, ELISA-measured microcystin concentrations declined by 4.8-fold, from 20 ppb to 4.2 ppb (Table 1). The rapid decline may be caused by (among other factors): wind-driven mixing, the photolabile nature of microcystins, and/or their metabolism by other microorganisms.[24,54,55] To investigate whether this temporal pattern exhibited by the ELISA data extended to the CSM mixture, the described suspect screening method was applied. We tentatively identified 18 CSMs, including two potential microcystins ([D-Asp3]MC-HilR [level 4] and MC-RR [level 2b]) and one potential anatoxin-a (11-carboxyanatoxin-a [level 4]), with no saxitoxins or nodularins identified. A complete list of tentatively identified features with associated information from the cyanometDB, critical acquisition results, and scored MS2 spectra when available, is provided in Tables S15–S18.

Although both ELISA and suspect screening data demonstrate an overall decrease in microcystin abundance over the sampling

Table 1 Microcystin concentrations and CSM feature normalized response at Lake Darling

		Time (hours)[a]						
		0.00	0.97	2.63	2.88	3.13	3.38	3.63
ELISA								
Microcystin (μg L^{-1})		19.98	8.64	4.16	5.72	6.20	5.45	4.18
Suspect screening								
Feature class[b]	**Tentative feature identification [conf. level]**	**Normalized log response[c]**						
Anatoxin-a	11-Carboxyanatoxin-a [4]	1	0.97	0.98	1.00	0.97	1.02	0.96
Microcystin	MC-RR [2a]	1	0.98	0.87	0.86	0.78	0.87	0.70
	[D-Asp3]MC-HilR [4]	1	0.96	0.83	0.88	0.77	0.88	0.76
Other cyclic non-peptide	Sacrolide A [3b]	1	0.98	0.90	0.88	0.90	0.88	0.87
	15,16-Dihydrosacrolide A [3b]	1	0.99	0.92	0.91	0.93	0.91	0.90
Other cyclic peptide	Tricholactone [3a]	1	0.98	1.00	0.98	0.99	0.95	0.98
	Palmyrrolinone [3a]	1	0.98	0.89	1.02	0.99	0.99	1.12
Other linear non-peptide	Koshikalide [3b]	1	1.00	1.01	1.01	1.00	1.01	0.99
	N-Butyryl-L-homoserine lactone [4]	1	0.99	0.98	0.97	0.95	0.97	0.97
	Lyngbyacarbonate [4]	1	1.02	1.02	1.03	1.00	1.04	1.00
	6-Acetamidotridecane [4]	1	0.98	1.01	1.01	0.88	0.98	0.80
	Palythene [3b]	1	1.08	1.18	1.20	1.13	1.16	1.14
	Gloeolactone [3b]	1	1.02	0.92	0.91	0.93	0.90	0.87
	7-Methoxy-9-methylhexadecadienoic acid [3b]	1	1.01	0.95	0.98	0.97	0.94	0.98
	Pukeleimide D [4]	1	0.98	0.94	0.94	0.92	0.96	0.92
	Puna'auic acid [4]	1	1.00	0.92	0.92	0.93	0.92	0.95
	Malyngic acid [3b]	1	1.01	0.94	0.92	0.95	0.91	0.93
	Microcystbiopterin A [4]	1	1.01	0.93	0.90	0.91	0.91	0.90
	478-Da MAA [3b]	1	1.00	0.91	0.91	0.91	0.89	0.90

Decrease / 0 / Increase

[a] Time series from 11:22 until 15:00 (t = 0 hours to t = 3.63 hours). [b] Feature class as listed in the CyanoMetDB. [c] Tentatively identified cyanometabolite features log response value normalized to t = 0 with boxes shaded according to results greater than response = 0 (blue) and less than response = 0 (red), with color intensity reflecting the magnitude of the deviation.

period (16 of the 18 features decreased overall), more detailed resolution revealed a much more dynamic system. Rapid up-and-down regulation (*i.e.*, increases or decreases, respectively, in relative abundance based on specific feature peak area) of the CSM features occurred, with 9 of the 18 features up-regulated between the first two sample events (Table 1). Specific features, such as one tentatively identified as palmyrrolinone [level 3a], experienced both up- and down-regulation in their responses over the course of sampling. With respect to the initial sample, the immediately following sample exhibited initially high similarity ($t = 1$ h; $\cos(\theta)_{\text{Darling}}$ 0.97); however, by the next sequential sample ($t = 2.6$ h), the similarity dropped ($\cos(\theta)_{\text{Darling}}$ 0.73) (see Table S19 for the full $\cos(\theta)_{\text{Darling}}$ matrix). The samples collected between 2.6 h and 3.4 h remained similar ($\cos(\theta)_{\text{Darling}} > 0.88$ between all samples). One final major shift occurred in the final sample ($t = 3.6$ h), with $\cos(\theta)_{\text{Darling}} < 0.76$ between the final sample and all earlier samples, likely due to the significant increase in the relative abundance of the tentatively identified CSM palmyrrolinone [level 3a], a dicraboximide with molluscicidal properties.[56] In contrast to the decrease in ELISA-measured microcystin concentration, the variability in trends observed in the feature mixture indicates potential cyanobacterial activity independent of cyanotoxin concentrations. These results demonstrate great temporal variability in the overall ELISA response and increases and decreases in the CSM complex mixture representation over a time period relevant to a recreationalist at a beach, with concomitant implications for highly variable exposure potential.

Beach monitoring implications

Iowa has a weekly beach monitoring program for all State Park beaches (41 unique sites) throughout the state, including the two test lake study sites, to help protect recreationalists from HABs and exposure to bacteria. The beach samples are composite samples from nine subsamples (three depths at three locations) along each beach, collected weekly from Memorial Day (late May) through Labor Day (early September) each year. Iowa's weekly beach monitoring program detected and reported microcystin concentrations of 1.23 ppb at Lake MacBride (August 16th, 2022) and 14.2 ppb at Lake Darling (August 9th, 2023), triggering an advisory only at Lake Darling but not Lake MacBride.[14] Nevertheless, our spatial and temporal sampling at those lakes revealed substantial discordance between weekly monitoring snapshots and potential exposure later in the same week (Fig. 2). This CSM variability observed at both lakes reveals limitations in current monitoring approaches.

At Lake MacBride, while weekly monitoring detected 1.23 ppb, our spatial sampling from the following day detected 36.89 μg L^{-1} at site South 2, a 30-fold greater value. Moreover, the spatial difference in microcystin concentration between the eastern and western ends of South Beach (36.86 μg L^{-1} and 0.65 μg L^{-1}, respectively) has implications for potential exposures, as beachgoers could experience concentrations well below and above the EPA advisory threshold despite being only 103 m apart and visiting during a no-advisory period. At Lake Darling, temporal data demonstrated substantial variability within a single monitoring period. The four-hour time-series concentrations dropped from 19.98 μg L^{-1} to 4.16 μg L^{-1} (from 2.5 times higher than the advisory threshold to approximately half the advisory threshold value) within 2.6 hours of the initial sampling. Beachgoers arriving in the late morning *versus* early afternoon would have experienced a 4.8-fold higher potential exposure to microcystins despite both periods being covered by

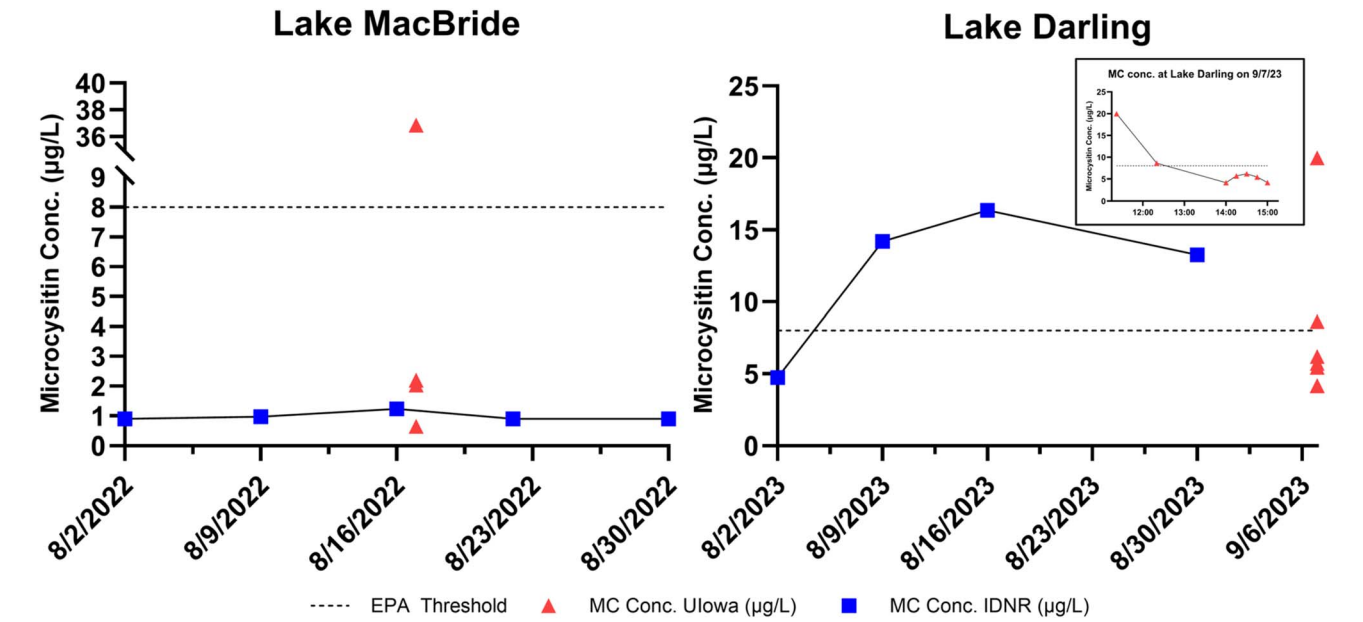

Fig. 2 Results from Iowa Department of Natural Resources (IDNR) beach monitoring[14] and the collected ELISA data for Lake MacBride and Lake Darling. The dashed line indicates the EPA advisory threshold for microcystin (8 μg L^{-1}). The inset figure under Lake Darling highlights the intraday timeseries for the UIowa collected samples (data also in Table 1).

the same weekly advisory, based on the 14.2 μg per L concentration detected by routine monitoring. Additionally, suspect screening indicates that HAB systems are dynamic beyond the well-studied cyanotoxins, underscoring the highly dynamic potential for exposure to HAB-associated CSM complex mixtures, posing greater challenges for modeling, monitoring, and public health communication efforts. Additional research to characterize HAB CSM spatiotemporal mixture dynamics using emerging methods such as HRMS suspect screening is warranted to inform exposure risk for water recreationalists.

Conclusions

In this work, we demonstrate the short-term spatiotemporal complexity of CSM complex mixtures during an active HAB event with novel suspect screening methods. With 38 unique tentatively identified CSMs from the two sites, each test site exhibited notable variability between samples ($\cos(\theta)_{MacBride\_min} = 0.375$ and $\cos(\theta)_{Darling\_min} = 0.515$) that would not have been captured using standard approaches. Although these approaches cannot explain the observed variability, the presented data highlight a critical need for further investigation into the drivers of sub-daily spatiotemporal dynamics of HAB events, which have not received substantial attention.[57]

Notably, CSM mixture characterization through suspect screening remains a developing approach for understanding HAB activity and dynamics. Although the CyanoMetDB is the most compressive database for CSMs, the lack of MS2 inherent in the data limits the ability of higher quality compound identifications. Indeed, there are MS2 spectra for CSMs that exist (and which were used for improving confidence of identification when available in databases and our samples; see SI), these spectra are split among several different databases, with many of the CSMs in the CyanoMetDB not present in commonly used databases like GNPS and mzVault. A more comprehensive spectral library is needed to support high-confidence identifications beyond *in silico* fragment matching. Improvements in identification confidence, while not the direct focus of this study, would help reduce potential error and could allow for a better understanding of the geographical distribution of CSMs. Importantly, this work shows that applying the CyanoMetDB as a NTA suspect screening approach could help reveal the underlying drivers and dynamics of HAB events that are unrecognized by conventional monitoring approaches. To the best of our knowledge, this work represents the first attempt to apply suspect screening for CSM in agroecosystem lakes specifically and should thus be viewed as an initial groundwork study quantitatively demonstrating complexity and bolstering the need for further investigation.

This work demonstrates that high spatiotemporal variation of CSM mixtures in agroecosystem lakes can alter the potential exposure faced by recreationists. HAB events need to be better understood across the extent of a lake and on sub-daily time scales to better characterize the variation in potential exposure during an active HAB event. Future work should further investigate the correlation between CSM mixtures and known drivers of bloom growth and degradation, employing suspect screening approaches in conjunction with remote sensing techniques to enhance the scope of future studies. This multi-faceted approach could help illuminate drivers of mixture change beyond what has been hypothesized. Future work should also consider pairing 16S rRNA sequencing and/or flow cytometry methods with suspect screening to better understand the linkages between cyanobacterial phylogenetic characterization and/or speciation with CSM mixture composition to better inform their relationships as well as broader distributions.[46,58] Moreover, further investigation into the impact of CSM mixtures on the actual risk posed to recreationists should be conducted, potentially using effect-directed analysis and direct CSM concentration measurements to both incorporate existing dose-response information and bolster suspect screening identification confidence.[20,44,59] The suspect screening for CSM in this work provides critical additional insight, illustrates the spatiotemporal variability in potential exposure, and lays the groundwork for understanding active HAB event dynamics in agroecosystem lakes.

Author contributions

Stanley W. Kohls: conceptualization, methodology, software, formal analysis, investigation, data curation, writing – original draft, visualization, project administration. Lyndy Holt: formal analysis, writing – review & editing. Nervana Metwali: formal analysis, writing – review & editing. Corey D. Markfort: writing – review & editing, funding acquisition. Peter S. Thorne: writing – review & editing, funding acquisition. Gregory H. LeFevre: conceptualization, methodology, resources, writing – review & editing, supervision, visualization, project administration, funding acquisition.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). The .raw files from the UPHLC-HRMS analysis are available from the corresponding author. Supplementary information: site and sample descriptions, analytical methods and chemicals, data processing, SI results figures/tables/statistics, and the NTA Study Reporting Tool (SRT). See DOI: https://doi.org/10.1039/d5em01071c.

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