In Vivo Phycocyanin Flourometry as a Potential Rapid Screening Tool for Predicting Elevated Microcystin Concentrations at Eutrophic Lakes

Jason W. Marion,[†] Jiyoung Lee,*,[†,§] J. R. Wilkins, III,[†] Stanley Lemeshow,[†] Cheonghoon Lee,[†] Evan J. Waletzko,[§] and Timothy J. Buckley[†]

[†]College of Public Health, Division of Environmental Health Sciences, and [§]Department of Food Science and Technology, College of Food, Agricultural and Environmental Sciences, The Ohio State University, 1841 Neil Avenue, Columbus, Ohio 43210-1351, United States

[‡]Olentangy River Wetland Research Park, 352 Dodridge St., Columbus, Ohio 43201, United States

⑤ Supporting Information

ABSTRACT: Current approaches for assessing human health risks associated with cyanotoxins often rely on the quantification of microcystin. Significant limitations of current approaches are cost and time to obtain a result. To address these challenges, a numerical index for screening microcystin risks above the World Health Organization's (WHO) low-risk threshold for microcystin was developed for eutrophic Midwestern U.S. lakes based on water quality results from 182 beach water samples collected from seven Ohio lakes. In 48 (26.4%) samples we observed microcystin concentrations as measured by ELISA that exceeded the 4 μg/L microcystin threshold. A multivariable logistic regression model using practical real-time measures of in vivo phycocyanin (by fluorometry) and secchi depth was constructed to estimate the probability of a beach sample exceeding 4 μg/L microcystin. The final model achieved statistical significance (p = 0.030) as well as good calibration (as measured by the goodness-of-fit test comparing observed to expected counts within deciles of risk based on the model, p = 0.329) and discrimination (as indicated by the area under the receiver-operator-curve (0.795)). These results demonstrate two rapid and practical measures of recreational water quality are effective in identifying "at risk" lake conditions warranting additional management (e.g., advisory and/or advanced testing).

■ INTRODUCTION

Harmful cyanobacteria are gaining substantial attention among water quality managers and public health officials around the world. This attention is motivated by our expanding knowledge of cyanotoxin toxicity and a growing body of evidence suggesting an increasing global trend in the frequency of harmful algal bloom (HAB) events.[1−3] Although not all cyanobacteria produce cyanotoxins, it is suspected that with increasing water temperatures and nutrient levels, conditions will be more favorable for cyanobacteria blooms that contain harmful genera.[4,5] There is some evidence suggesting warmer water temperatures will create selective pressures enabling genera with potentially harmful species, such as *Microcystis*, to exhibit dominance over entirely nonharmful genera in freshwater U.S. lakes.[6] The public health concern for harmful

cyanobacteria is warranted, as they are known to produce a variety of cyanotoxins that act as potent hepatotoxins, neurotoxins, cytotoxins, irritants, and/or gastrointestinal toxins.[7,8]

Adverse health threats associated with cyanobacteria were first observed in animals in 1878.[9] More recently (1996) microcystin and cylindrospermopsin were implicated in the deaths of 76 humans in Brazil.[10] Beyond these two reports, epidemiologic studies conducted in China have linked microcystin exposure to increased liver cancer incidence.[11] With respect to recreational exposure, the epidemiology is limited to a handful of cohort studies conducted in Australia[12−14] and the U.S.,[15] and the case reports that exist[16] largely rely on cell densities as opposed to toxin concentrations.

Despite the limited epidemiologic evidence, the World Health Organization (WHO), established provisional drinking and recreational water guidelines relying on toxicity studies in mice and pigs.[17,18] In contrast, the U.S. Environmental Protection Agency (EPA) recently concluded that the information presently available is insufficient for guiding U.S. policies pertaining to cyanobacteria.[19] Among U.S. states, Nebraska, New York, Florida, and Ohio all have recently issued numerous human contact advisories for freshwater environments relying on the WHO guidelines.[20−23] Derived from the drinking water provisional guideline for microcystin that was set at 1.0 μg/L,[24] a "moderate" microcystin health risk guideline of 20 μg/L (~100 000 cyanobacteria cells/mL) for recreational waters was established by WHO.[25] The "low" health risk range was derived from human epidemiologic and mouse model[17] studies, resulting in recommended advisory signage at 20 000 cyanobacteria cells/mL,[25] which approximates to 4 μg/L of microcystin. This low health risk range is presumed to be associated with an increased likelihood of short-term adverse health outcomes, such as skin irritations and gastrointestinal illness (GI), compared to the no recognized health risk level. The posting of advisory signage is recommended at this level and increased surveillance is encouraged by WHO.[24,25] Chlorophyll *a* concentrations of 10 μg/L and 50 μg/L serve as alternative measures for issuing low and moderate risk advisories respectively when cyanobacteria dominate.[25]

Given the high relative abundance of microcystin compared to other toxins,[8] a substantial amount of research on identifying harmful blooms has focused on microcystin. Rapid methods such as microcystin-specific ELISAs have been developed,[26] improved upon,[27] and are now commonly applied for research and regulation.[24,28,29] Newer approaches, such as quantitative polymerase chain reaction (qPCR) amplification of toxin-producing synthetase genes[30,31] and ELISA-based sensor applications for estimating microcystin concentrations have gained attention.[32] Collectively, these methods provide substantial improvement in estimating microcystin concentrations; however, practical limitations pertaining to laboratory resources, sample transport, timeliness of reporting, and cost remain a concern.

An alternative nonmolecular approach involves methods for identifying harmful blooms that focus on quantification of phycocyanin pigment via flourometry.[33] Recently, it was demonstrated in inland northern U.S. waters that flourometric procedures focusing on phycocyanins were not only associated with cell densities, but also with microcystin concentrations.[34−36] To address the public health need for a rapid and practical means for predicting microcystin concentrations, the goals of this study were to (1) evaluate the predictive association between two rapidly measured pigments, that is, in vivo phycocyanin (PC) and chlorophyll *a*, and microcystin concentrations at seven inland Ohio beaches, and (2) develop a practical approach and model for beach management for the protection of public health. The resulting approach and model provides a unique and practical tool for screening beach waters for the rapid determination of potentially elevated and health-relevant microcystin concentrations.

■ EXPERIMENTAL SECTION

Sampling Sites. We evaluated beach water quality at seven Ohio inland lakes from central and southern Ohio (Figure 1).

Figure 1. Map of Ohio, United States, showing the locations of the lakes used in this study.

More detailed location information is available (see Supporting Information (SI) Table S1). A total of 26 samples were collected from each beach during the 2009 swimming season (May 28 to August 30). Five beaches were located on reservoirs operated by the U.S. Army Corps of Engineers primarily for flood control with a secondary recreational use designation. The beaches at these five Corps lakes attract over 575 000 swimmers annually.[37] The other two lakes are state-owned recreation reservoirs (swimmer usage data not available). The trophic statuses of the lakes range from mesotrophic to hypereutrophic,[38] with most lakes being classified as eutrophic; the Corps lakes are substantially larger than the state-owned lakes (see SI Table S1).

Sampling Procedure. The sampling methodology employed is described in Marion et al.[39] In brief, samples were collected biweekly from the public beaches at the seven study lakes in accordance with Ohio Department of Health standard methods.[40] All analyses performed on the beach water were generated from these single twice weekly samples. Field observations of weather and real-time measurements of water quality were recorded in conjunction with sample collection. From each sample, approximately 12 mL of water was stored at −80°C in 15 mL plastic centrifuge tubes for later analysis of microcystins. To enable PCR analysis from three lakes, additional water samples were first prefiltered through a large

Table 1. Beach Water Quality Characteristics Across Seven Inland Ohio Reservoirs over 26 Sampling Days during Summer 2009

	lake abbreviation						
	$n = 26$	$n = 26$	$n = 26$	$n = 26^h$	$n = 26$	$n = 26$	$n = 26$
parameter (mean ± S.E.)	AC[a]	BC[b]	DC[c]	DEL[d]	EF[e]	LL[f]	MAD[g]
temperature (°C)	25.4 ± 0.38	25.4 ± 0.41	26.0 ± 0.33	25.8 ± 0.38	26.7 ± 0.36	27.0 ± 0.41	25.9 ± 0.31
specific conductivity (µS)	432 ± 1.86	435 ± 10.5	459 ± 7.39	474 ± 3.96	276 ± 2.29	209 ± 5.31	486 ± 10.9
dissolved oxygen (mg/L)	9.72 ± 0.66	11.6 ± 0.78	11.9 ± 0.67	12.1 ± 1.06	10.2 ± 0.51	10.4 ± 0.51	13.0 ± 1.21
pH	8.48 ± 0.33	8.66 ± 0.02	8.85 ± 0.03	8.67 ± 0.04	9.10 ± 0.04	8.82 ± 0.05	8.73 ± 0.05
secchi depth (cm)	118 ± 6.37	68.7 ± 1.70	54.5 ± 2.56	53.1 ± 2.22	62.1 ± 3.09	72.0 ± 2.43	34.3 ± 1.06
total phosphorus (mg/L)	35.9 ± 6.62	64.6 ± 4.26	90.8 ± 5.20	95.4 ± 5.64	107 ± 11.2	55.3 ± 3.40	210 ± 8.20
chlorophyll A (mg/L)	4.67 ± 0.40	22.2 ± 1.31	13.5 ± 1.20	25.7 ± 2.20	16.6 ± 1.78	12.3 ± 0.78	67.7 ± 9.10
turbidity (NTU)	6.15 ± 0.51	10.0 ± 0.75	20.5 ± 1.56	18.3 ± 1.70	22.6 ± 4.70	9.15 ± 0.57	40.7 ± 4.18
mean trophic state index	51.3 ± 1.03	63.3 ± 0.38	64.3 ± 0.65	66.8 ± 0.61	64.9 ± 0.73	60.3 ± 0.54	75.4 ± 0.58
phycocyanin (µg/L)	6.04 ± 1.18	73.7 ± 3.41	160 ± 13.1	59.9 ± 8.15	57.1 ± 4.08	28.1 ± 1.35	130 ± 15.8
no. samples ≥4 µg/L microcystin (%)	6 (23.1)	8 (30.8)	18 (69.2)	4 (15.4)	0 (0)	3 (11.5)	9 (34.6)
no. samples positive for *M. aeruginosa* (PC-IGS) (%)				9/21 (42.9)	23/23 (100)		6/21 (28.6)
no. samples positive for *M. aeruginosa mcyA* gene (%)				6/21 (28.6)	14/23 (60.9)		2/21 (9.52)

[a]Alum Creek Lake. [b]Buck Creek Lake. [c]Deer Creek Lake. [d]Delaware Lake. [e]East Fork Lake. [f]Lake Logan. [g]Madison Lake. [h]Secchi Depth $n = 25$.

pore filter (20 µm pore size, 47 mm diameter; Magna, GE Water & Process Technologies, Trevose, PA) to remove algae and other debris that may interfere with PCR results. Each water sample (500 ml for EF, and 200 ml for DEL and MAD) was subsequently filtered through a 47 mm diameter membrane with a 0.45 µm pore size. Filter membranes were placed in 50 mL tubes and stored at −80°C until further processing.

Water Quality Indicators. Temperature, pH, specific conductivity and dissolved oxygen were recorded at the time of sampling using a multisensor probe (YSI 600XL multiprobe data sonde (Yellow Springs, Ohio)). Total phosphorus was quantified with the acid persulfate digestion method using Hach Method 8190 (Hach Company 2007[41]) and a Hach DR2800 spectrophotometer (Hach, Loveland, Colorado). Turbidity was quantified using the Hach 2100P portable turbidimeter.[41] Secchi depth (SD) or transparency was measured using a submerged secchi disk.[42] Due to thunderstorm activity, secchi depth was not measured for one lake sample. Chlorophyll *a* and PC were both quantified in vivo, using the intact cells without filtration or extraction. Both chlorophyll *a* and PC were quantified using a two-channel hand-held Aquaflour flourometer (Turner Designs, Sunnyvale, California). The simple flourometry procedures used in this study were as follows: Using a pipet, 4 mL of sample water was placed into a Turner Designs methacrylate cuvette. The filled cuvette was placed in the flourometer and using channel A, the relative units for PC were recorded. Without removing the cuvette from the instrument, channel B was selected and relative units for chlorophyll *a* were recorded. Chlorophyll *a* (excitation at 460 ± 20 nm, emission >665 nm) was standardized ($R^2 = 99.9\%$) with liquid primary chlorophyll *a* standards (catalog number 10-850, Turner Designs). PC (excitation at 595 nm, emission at 670 nm) was standardized ($R^2 = 99.9\%$) using C-phycocyanin standard from *Spirulina* sp. (catalog number P6161, Sigma-Aldrich, St. Louis, Missouri). The mean trophic state index (TSI) was calculated by averaging the TSI estimate from total phosphorus, secchi depth, and in vivo chlorophyll *a*. The mean TSI calculation differs from Carlson's TSI[43] in that it used in vivo chlorophyll *a* as opposed to extracted chlorophyll *a*.

Microcystin Detection and Quantification. The concentration of microcystin was measured using the EPA-validated Microcystins/Nodularins (ADDA) ES ELISA kit in 96-well format (catalog number PN520011ES, Abraxis, Warminster, Pennsylvania).[44] The samples were thawed and then underwent a freeze/thaw procedure two times at −20°C to effectively rupture cells and release the toxins.[36] After final thawing, samples were used in the ELISA per manufacturer instructions and evaluated using a plate photometer (Dynex Technologies MRX TC Revelation). The assay detects and quantifies numerous variants of currently known microcystins with polyclonal antibodies providing a concentration for total microcystins in microcystin-LR equivalents (MC-LR eq.).[44] For each multiwell plate, a total of six standards were used in duplicate for developing the plate-specific standard curves (mean $R^2 = 98.0\%$, R^2 range = 96.2−99.1%). Additionally, positive and negative controls were used, and all samples were evaluated in duplicate. There was good agreement between the two measured optical densities for each sample (Pearson correlation = 0.966, $p < 0.001$). Samples exceeding 5 µg/L MC-LR eq. exceeded the assay's specified range of detection. Samples below 0.15 µg/L were below the specified range of quantification. Samples below 0.10 µg/L were below the specified limit of detection.

Real-Time PCR Assays. Following DNA extraction from the filters, real-time PCR targeting the phycocyanin intergenic spacer (PC-IGS) for *M. aeruginosa* detection and the *M. aeruginosa*-specific microcystin synthetase gene, *mcy*A was carried out with a StepOne Real-Time System (Applied Biosystems, Foster City, CA) in a 48-well format with a total reaction volume of 20.0 µL using a mixture containing 2.0 µL of DNA, each forward and reverse primer (500 nM for PC-IGS and 200 nM for *mcy*A detection; SI Table S2) and 10.0 µL of SYBR universal PCR master mix (Applied Biosystems). Details regarding the positive control strain, DNA extraction methods, PCR conditions, limit of detection determination, and generation of the standard curve are provided in the Supporting Information. For statistical analyses using PCR results, nondetects were replaced with the lower limit of detection.

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Data Analysis. Data were initially explored using scatterplots and regression analysis for continuous data. Logistic regression was used with 0/1 coding for the event variable, where a positive event was defined as any sample exceeding 4 μg/L MC-LR eq., which corresponds to the WHO low health risk range of 20 000 cyanobacteria cells/mL.

Modeling was performed taking into account clustering due to the repeated measures from each lake. Numerous models were constructed using investigator-controlled backward selection procedures. Using this backward selection procedure, covariates with Wald test p-values < 0.15 were considered for the full model.[45] After including all covariates into a full model, the least significant terms were individually removed, one at a time, until the most parsimonious and statistically significant model could be obtained. For the multivariable models, continuous covariates were evaluated for linearity in the logit via the fractional polynomial method.[46] For covariates observed to be nonlinear in the logit, appropriate transformations were used.

■ RESULTS AND DISCUSSION

Characterization of Beach Water Quality. Water quality characteristics at the seven inland lakes are described in Table 1. The water quality observed in our study was consistent with previous observations by Ohio EPA.[38] The trophic state index places all the lakes except Madison Lake in the eutrophic classification, which was observed to be hypereutrophic. Alum Creek, characterized as mesotrophic by many measures in 1996 also was characterized in some reports as being mildly eutrophic, a condition which we observed with a mean TSI of 51.3.

Among microcystin samples, a total of 43 (23.6%) samples exceeded the upper detection limit of 5 μg/L MC-LR eq. These samples had significantly higher PC measurements (median = 103.2 μg PC/L) than the other 139 samples (median = 48.6 μg PC/L) according to the Mann–Whitney Test (p < 0.001). These results are not surprising as Izydorczyk et al.[47] also observed higher PC levels when microcystins exceeded 5 μg/L in their study of a Polish reservoir.

The analysis comparing median lake concentrations of PC and the percent of samples exceeding 4 μg/L MC-LR eq. (Figure 2) demonstrated a positive association (r = 0.82, Pearson p = 0.025), whereas no association was observed when

Figure 2. The association of median in vivo phycocyanin concentrations and the percent of samples exceeding the low risk threshold for microcsytins (4 μg/L) in the Ohio lakes during the study period.

performing the same analysis between median chlorophyll a by lake and percent of microcystin samples exceeding the low risk threshold (r = 0.16; Pearson p = 0.733). No other associations (based on Pearson correlation p-values ≤ 0.05) with microcystin levels and other parameters such as sampling month, water temperature, pH, turbidity and specific conductivity were observed. A similar association between microcystin and PC concentrations was observed by Izydorczyk et al.[47] at Sulejów Reservoir in Poland (r = 0.51, n = 31, p < 0.05). Other studies have examined the association between PC and *Microcystis* spp. cell density. In the Ahn et al.[48] study of a large Korean reservoir, the correlation between PC and *Microcystis* spp.(r = 0.80, n = 25, p < 0.001) was stronger than between chlorophyll a and *Microcystis* spp. (r = 0.58, n = 25, p < 0.01). Ahn et al.[48] observed additional significant associations between *Microcystis* spp. and a variety of nitrogen and phosphorus-related parameters. Our study differs from those described above in two important ways. First, we measured microcystin directly allowing measures of associations to be performed with the percentage of samples exceeding 4 μg MC-LR eq./L versus *Microcystis* spp. cell density. Additionally, our results also pertain to several reservoirs versus one.

Real-Time PCR Results. DNA extracts were obtained from Delaware (n = 21), Madison (n = 21) and East Fork (n = 23) lakes. PC-IGS genes that target *M. aeruginosa* was detected in all samples at EF (100%), and were less frequently observed at Delaware (42.9%) and Madison (28.6%). With regards to positive detections of *M. aeruginosa* capable of producing toxin (possessing *mcy*A), this subpopulation was observed most frequently at East Fork (in 60.9% of samples), whereby, it only occurred in 28.6% of DEL samples and 9.52% of Madison samples. No significant association was observed between the copy number of the *mcy*A gene or PC-IGS with microcystin concentrations. Among 23 samples at East Fork, no samples exceeded 4.0 μg/L MC-LR eq., despite East Fork having the most frequently observed PC-IGS and *mcy*A detections. In total, out of the 65 samples across the three lakes with DNA extracts, 11 samples (16.9%) had MC-LR concentrations exceeding 4.0 μg/L. Only one sample of these 11 (9.09%) had detectable *mcy*A gene copies. Only three of these 11 (27.3%) had detectable gene copies of *M. aeruginosa*-specific PC-IGS.

Univariable Logistic Regression. Univariable models were considered before multivariable models. Among 12 water quality indicators (see Table 2), only three demonstrated some association (p < 0.15) with elevated microcystin levels (specific conductivity, dissolved oxygen, and PC as determined using the Wald statistic). An evaluation of these crude models demonstrated that PC is superior as an individual indicator.

Using the area under the receiver-operating-characteristic (ROC) curve to assess discrimination of each significant crude model, PC demonstrated acceptable discrimination (73.3%), whereas dissolved oxygen exhibited poor discrimination (58.8%). Specific conductivity performed similarly to PC with respect to discrimination (area under ROC curve = 69.4%). An assessment to determine whether the continuous variables in the model were linear in the logit was made via the fractional polynomial method.[46] This assessment suggested that PC does not appear to be related to elevated microcystin as a linear function in the logit. This was demonstrated by the deviance associated with this single term which was significantly higher than the deviance by the best second order polynomial (p <
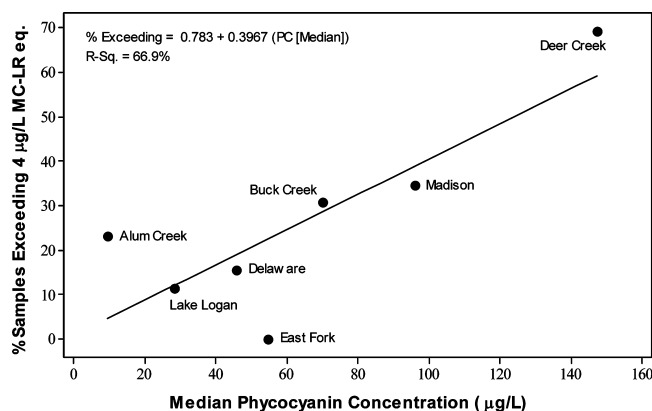
Table 2. Univariable Logistic Regression Results for Lake Clustered Data Across Across Seven Inland Ohio Reservoirs over 26 Sampling Days during Summer 2009 with MC-LR eq. > 4 μg/L Set As the Outcome Variable

univariable models	OR (95% CI)[b]	SE $_{OR}$[c]	Wald (p)
temperature (°C)	0.94 (0.75−1.15)	0.080	0.470
specific conductivity (μS)	1.01 (1.00−1.02)	0.003	**0.040**
dissolved oxygen (mg/L)	1.08 (1.00−1.16)	0.032	**0.047**
pH	0.60 (0.03−12.8)	0.694	0.694
secchi depth (cm)	0.99 (0.97−1.02)	0.010	0.585
total phosphorus (μg/L)	1.00 (1.00−1.00)	0.001	0.943
in vivo chlorophyll A (μg/L)	0.99 (0.99−1.01)	0.827	0.827
turbidity (NTU)	1.00 (0.98−1.03)	0.009	0.780
trophic state index (mean)	1.02 (0.98−1.05)	0.014	0.302
in vivo phycocyanin (μg/L)	1.01 (1.00−1.03)	0.005	**0.053**
Microcystis aeruginosa (copies/100 mL)[a]	0.99 (0.99−1.00)	0.001	0.398
M. aeruginosa-specific *mcy*A (copies/100 mL)	0.99 (0.99−1.00)	0.001	0.357

[a]*Microcystis aeruginosa* PC-IGS copies. [b]95% Confidence Interval of odds ratio. [c]Standard Error of Odds Ratio.

0.001). Given this observation, a second order polynomial transformation (see Table 3) was used.

Table 3. Final Multivariable Logistic Regression Model for Predicting the Odds of Beach Samples Exceeding MC-LR eq. Concentrations of 4 μg/L in Ohio Reservoirs

covariate	β	SE $_b$	Wald (p)	OR (95% CI)
in vivo phycocyanin (μg/L)	0.0507	0.0108	0.003	1.05 (1.02−1.08)
in vivo phycocyanin^2 (μg/L)	−0.0001	0.0000	0.008	1.00 (1.00−1.00)
secchi depth (cm)	0.0300	0.0051	0.001	1.03 (1.02−1.04)
constant term	−5.9812			

Multivariable Logistic Regression. A multivariable model initially containing all three of the significant covariates (i.e., dissolved oxygen, specific conductivity, and PC, $p < 0.15$) from the univariable models was constructed. The dissolved oxygen term was associated with the highest p-value ($p = 0.131$) and was therefore removed from the model resulting in a two-variable model that included specific conductivity ($p = 0.108$) and PC ($p = 0.100$). PC was determined to be nonlinear in the logit and it was decided to include its squared term in the model. Using the model terms (PC and PC^2), specific conductivity became less significant ($p = 0.177$) and was therefore removed. Individually, additional covariates were added to construct the most significant model as determined by the likelihood ratio test, while ensuring acceptable discrimination and model fit. The covariates turbidity ($p = 0.017$), SD ($p = 0.012$), and mean TSI ($p = 0.004$) enhanced model performance when included in the model individually. The model containing SD was determined to provide the most discrimination and had the best fit according to the Hosmer-Lemeshow goodness-of-fit test.

Overall, the logistic model containing PC (μg/L), PC^2 (μg/L)^2, and secchi depth (SD (cm)) provided the best model for estimating the probability of elevated MC-LR eq. levels as determined by the Likelihood Ratio Test ($p = 0.0298$) (see Table 3). Because no interaction was observed between secchi

depth and PC ($p = 0.511$), the resulting model was more useful and easily interpreted. Using the coefficients provided in Table 3, the probability of MC-LR eq. concentrations exceeding 4 μg/L can easily be calculated using only PC (μg/L) and SD(cm) values. First logit(p) is calculated using eq 1. An illustrative applied example of eq 1 using the median PC and SD results of our study is shown in eq 2.

$$\text{logit}(p) = \text{constant} + \beta_1(\text{PC}) + \beta_2(\text{PC}^2) + \beta_3(\text{SD}) \tag{1}$$

$$\begin{aligned} \text{logit}(p) &= -5.9812 + 0.0507(60.1) - 0.0001(3612.0) \\ &\quad + 0.0300(62.0) \\ &= -1.4353 \end{aligned} \tag{2}$$

Once logit(p) is determined, the probability of a sample exceeding the 4 μg/L MC-LR eq. concentration threshold is calculated using eq 3 and exemplified in eq 4 resulting in a probability of 19.2% when PC = 60.1 μg/L and SD = 62.0.

$$p = e^{\text{logit(p)}}/(1 + e^{\text{logit(p)}}) \tag{3}$$

$$p = e^{-1.4353}/(1 + e^{-1.4353}) = 0.1922 \tag{4}$$

Evaluation of Model Performance. The predictive capability and diagnostic accuracy of a test can be comprehensively evaluated through receiver operating characteristic (ROC) analysis.[49] This approach was used to evaluate the final multivariable model containing PC (μg/L), PC^2 (μg/L)^2, and SD (cm) and indicated very good discrimination where an area under the ROC curve of 79.5% was observed (SI Figure S1). Additionally, model calibration via the Hosmer-Lemeshow goodness of fit test indicated that the probabilities produced by the multivariable model reflected the true outcomes experienced in the data across the deciles of risk ($p = 0.33$).

Relevance to Beach Management. The application of the proposed screening method for beach management requires consideration of its strengths and limitations. The method's strengths primarily relate to its practical advantages of implementation, that is, measurements are relatively easily and inexpensively accomplished and results are instantly available. For example, the instruments to measure PC and SD cost approximately U.S. $2300 and $25, respectively, and can be easily and repeatedly used by field staff with only a few hours of training providing immediate results with no analysis costs. Once the measurements are made, the probability calculations could be easily accomplished within a spreadsheet set up for this purpose or through an index so that the beach manager would simply enter the PC and SD values and the probability would be automatically calculated. The more difficult issue relates to defining the appropriate action for a given probability given model limitations of sensitivity, specificity, and uncertainty. There are a wide range of actions available to beach managers including doing nothing, conducting additional monitoring, and/or closing the beach. Because this model was constructed based on a conservative microcystin (MC-LR eq.) value of 4 μg/L (in comparison to the 20 μg/L), the model application is more appropriately oriented toward screening (e.g., triggering additional sampling) than decisions of significant social and economic impact (e.g., beach closure). In this context, we propose a threshold probability (p) of 0.26. This threshold probability was selected

Table 4. Model Performance Assuming >26% Probability of Exceeding 4 μg/L Microcystin (MC-LR eq.) Evaluated Across Varying Secchi Depths[a]

deciles of secchi depth (cm)	N	calculated phycocyanin threshold (μg/L)	correct above pc threshold (sensitivity %)	correct below pc threshold (specificity %)	false positives	false negatives	positive predictive value %	negative predictive value %	correctly classified %
32	21	98	4 (57.1)	8 (57.1)	6	3	40.0	72.7	57.1
41	19	90	9 (100)	6 (60.0)	4	0	69.2	100	78.9
47	15	84	5 (100)	6 (60.0)	4	0	55.5	100	73.3
54	19	78	4 (80.0)	10 (71.4)	4	1	50.0	90.9	73.7
61	19	72	3 (100)	13 (81.3)	3	0	50.0	100	84.2
65	17	69	2 (40.0)	9 (75.0)	3	3	40.0	75.0	64.7
70	18	65	5 (100)	9 (69.2)	4	0	55.6	100	77.8
76	17	60	0 (0.0)	11 (78.6)	3	3	0.0	78.6	64.7
87	18	52	1 (100)	14 (82.4)	3	0	25.0	100	77.8
124	18	26	1 (20.0)	12 (92.3)	1	4	50.0	75.0	72.2
total	181	varies	34 (70.8)	98 (73.7)	35	14	49.3	87.5	72.9

[a]Phycocyanin is calculated at the 26% probability threshold and compared to observed MC-LR concentration classifications from 13 Ohio reservoirs.

as it balances the sensitivity and specificity of the screening model proposed. The selection of a probability cutpoint of 0.26 optimizes both sensitivity and specificity as appropriate for beach management decision-making aimed at protecting beach users from moderate health risks associated with microcystin exposure ($\geq$4.0 μg MC-LR eq./L). To visualize the relationship, see SI Figure S2, which demonstrates that as the probability increases for a sample to exceed $\geq$4.0 μg MC-LR eq./L, the model specificity also increases. As expected, as the probability increases, the sensitivity of the model decreases. If we were determining thresholds for other models designed for rapidly predicting more hazardous conditions (microcystin concentrations >20 μg/L), a more conservative approach that maximizes sensitivity even at the cost of specificity would be justified.

If the probability (p) exceeds 0.26, we recommend management action of an informational advisory and additional confirmational laboratory analysis. Table 4 demonstrates that this threshold accurately predicts 49% of the water samples with elevated microcystin risks (positive predictive value). Furthermore at this same cutpoint, the model accurately determines 88% of the samples when MC-LR eq. concentrations are below 4.0 μg/L. By adjusting the probability cutpoint to a more conservative figure (any value <0.26), model sensitivity and the positive predictive value would improve; however, model specificity and the negative predictive value would decrease. Additionally, by using a more conservative cutpoint, the number of samples exceeding the management action probability would increase, potentially increasing the amount of advisories or samples being submitted for laboratory analysis. A summary of the sensitivity and specificity for the model using the probability cutpoint proposed (p = 0.26) is depicted in Table 4. A quick analysis of Table 4 demonstrates the impact of secchi depth on maximum allowable PC concentrations, which can vary between 26 and 98 μg/L. Furthermore, it is apparent that in the more transparent waters (high SD values), the model loses positive predictive capability. Accordingly, there is some indication that our model may have more limited predictive validity for mesotrophic and oligotrophic waters, although our sample size is quite limited for these lakes providing justification for additional studies.

Microcystin Screening Index. To simplify the determination of an "action level" based on the approach described above for potential use by beach managers, we have developed the Microcystin Screening Index (MSI) applicable to eutrophic lakes. The MSI can be readily calculated from eq 5 and related to the exceedance probability distribution function using Figure 3. The previously determined and justified probability threshold
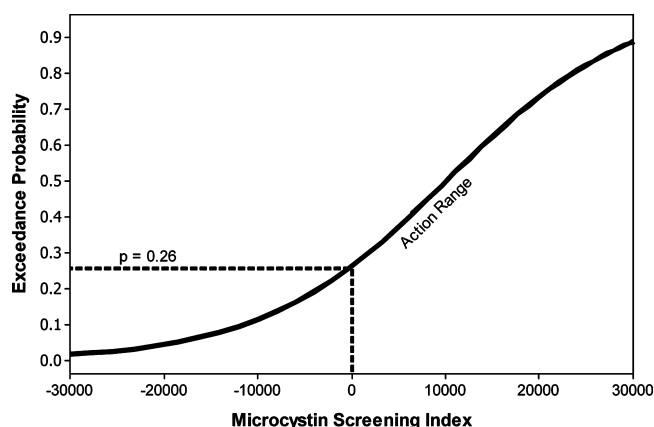
Figure 3. A plot illustrating the microcystin screening index (MSI) and the associated exceedance probability for a sample to exceed 4 μg/L (MC-LR eq.) with the action level denoted for MSI values plotted beyond the exceedance probability threshold of 0.26.

of 0.26 is set to zero such that MSI > 0 fall within the "action warranted" range where action may consist of posting an advisory and/or conducting additional testing. The applied example of eq 5 using the median PC and SD results of our study is illustrated in eq 6.

$$\text{MSI} = 500(\text{PC}) - \text{PC}^2 + 300(\text{SD}) - 49000 \qquad (5)$$

$$\text{MSI} = 500(60.1) - (60.1)^2 + 300(62.0) - 49000$$
$$= -3962 \qquad (6)$$

In this example, by plotting the MSI score on the graph presented in Figure 3, it is apparent that the risk for elevated microcystin concentrations is low, and therefore no action

would be warranted. By using Figure 3, the estimated probability of observing elevated microcystin (MC-LR eq.) concentrations can be easily and quickly determined.

Phycocyanin for Predicting Microcystin Concentrations. Our observation of a stronger association between microcystin and PC than microcystin versus all other parameters is consistent with the study results reported by Rinta-Kanto et al.[50] In their Lake Erie studies, PC had the highest Spearman correlation coefficient with microcystin (r_s = 0.76, n = 45, $p <$ 0.001), outperforming three genetic markers and 12 limnological parameters, including extracted chlorophyll a (r_s = 0.66, n = 85, $p <$ 0.001). Samples taken from the more nutrient- and toxin-rich western basin of Lake Erie, also showed a significant Spearman correlation (r_s = 0.63, n = 25, $p <$ 0.001). Only percent cyanobacteria as *Microcystis* spp. proved to be a stronger predictor (r_s = 0.70, n = 37, $p <$ 0.001). There are important differences in study approach that limit our ability for comparison. First, we quantified microcystin by ELISA rather than PPIA. Second, we treated microcystin as a binary variable (above or below 4 μg MC-LR eq. /L) and used logistic regression to build a predictive model. Third, we quantified PC using a rapid in vivo fluorometric method rather than filtration and extraction. Fourth, our samples came from multiple human-made freshwater lakes versus Lake Erie. Fifth, our samples represented nearshore waters at a depth of 0.3 m versus pelagic zone sampling occurring at variable depths. Despite these differences in approach, we see striking consistencies in PC as a valuable predictor of the microcystin toxin.

The results are entirely biologically plausible as in vivo PC has been demonstrated to be very effective in estimating total cyanobacterial biovolume when this biovolume exceeds 1 mm^3/L, which is ideal for rapidly monitoring cyanobacteria blooms.[51] Similar to our findings and others,[50] Zamyadi et al.[52] also demonstrated the superiority of the PC measurement over chlorophyll a for monitoring potentially harmful cyanobacteria. Our recommendation to use PC for rapidly assessing beach conditions is in line with recent recommendations for drinking water plants to use real-time PC measurements for screening cyanotoxin risks in source waters.[53] In none of these studies or in this study, is the PC measurement to be used in a manner that supplants water quality manager judgment.

Although PC monitoring is superior to other approaches for the real-time prediction of elevated microcystin levels, the approach does have limitations. As demonstrated in our model, water transparency (measured by SD) should always be measured. Others have demonstrated that similar to transparency, water turbidity can significantly influence PC results. More examples of potential sources of interference such as the health of the cyanobacteria cells, phycocyanin production per cell, the presence of phycobilin protein-possessing cryptophytes, and other potentially interfering factors are described by Zamyadi et al.[52] Another potentially confounding matter pertains to microcystin persistence in the water, which has been demonstrated to be variable across different aquatic environments. This is important to note as very important post-bloom dynamics between residual PC and microcystin may be influenced by a variety of lake-specific factors such as dilution factors that relate to the size and overall water quality of the lake.[54] In examining the scientific literature, it is suggested that increasing secchi depth (more transparent water) is generally associated with a lower likelihood for harmful microcystin concentrations;[55] however, our multivariable model failed to

confirm this phenomenon. Although our data supports the interpretation that an increasing secchi depth is negatively correlated with PC concentrations (r = −0.539, $p <$ 0.001), the more transparent water is associated with a greater probability for advisory level microcystin concentrations when there are increases in PC concentrations. This observation is biologically plausible as background levels of PC at more turbid, low secchi depth lakes are likely to be higher, suggesting greater competition among cyanobacteria. However, in more transparent lakes, where background PC levels are low, a small increase in PC is potentially associated with rapidly growing bloom-formers in response to favorable conditions and less competition.

In summary, the positive association between cyanobacteria, including specifically *Microcystis* cell density, and PC pigment has been well documented,[33,48,56] and has also been shown to outperform the chlorophyll a quantification methods recommended by the WHO to predict harmful blooms.[24,25] The results of this study demonstrated the potential value of predictive models for rapid and practical screening of elevated microcystin risk at inland beaches using PC coupled with SD. This study focused on predicting water quality events exceeding the "relatively mild and/or low probability of adverse health effects" risk level of 4 μg/L MC-LR eq., which approximates to 20 000 cyanobacteria cells per mL using WHO assumptions.[24] This study did not focus on samples exceeding the "moderate probability of adverse health effects" risk level of 20 μg/L microcystin, which recommends restricting bathing. Future studies evaluating the association of PC and higher microcystin levels than those evaluated in this study are warranted. Studies examining the association of PC and microcystin at inland beaches in other geographic areas and in lakes with other trophic statuses are encouraged as well as studies that examine this association beyond the swimming season. Lastly, this study again reconfirms the results of several studies[34−36] that demonstrated a significant association between microcystin and PC. Beach managers and regulatory entities should consider this phycocyanin-based approach as a potential screening tool for the rapid estimation of microcystin risk for the protection of human health in recreational waters when immediate sample results are not available.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: (614) 292-5546; e-mail: jlee@cph.osu.edu.

Notes

The authors declare no competing financial interest.

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After the paper was published ASAP on March 21, 2012, additional scientific corrections were made in the Experimental, Results, and References sections. The revised version was reposted on March 27, 2012.

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