PERFORMANCE DEMONSTRATION STATEMENT
MBio HAB Toxin MC/CYN Gen 1 System

TECHNOLOGY TYPE:	Algal Toxin Detection Field Kit
APPLICATION:	Field estimates of algal toxins for coastal environments
PARAMETERS EVALUATED:	Accuracy, precision, range response and reliability
TYPE OF EVALUATION:	Laboratory and Field Performance Demonstration
DATE OF EVALUATION:	Testing conducted from May 2018 to October 2018
EVALUATION PERSONNEL:	T.H. Johengen, H. Purcell, G.J. Smith, H. Bowers, D. Schar, T. Davis, M. Tamburri, D. Fyffe, and E. Buckley.

NOTICE:

ACT Demonstration Projects are evaluations of technology performance under specific, agreed-upon protocols, criteria, and quality assurance procedures. ACT and its Partner Institutions do not certify that a technology will always operate as demonstrated and make no expressed or implied guarantee as to the performance of the technology or that a technology will always, or under circumstances other than those used in testing, operate at the levels demonstrated. ACT does not seek to determine regulatory compliance; does not rank technologies nor compare their performance; does not label or list technologies as acceptable or unacceptable; and does not seek to determine "best available technology" in any form. The end user is solely responsible for complying with any and all applicable federal, state, and local requirements.

This document has been peer reviewed by ACT Partner Institutions and a technology-specific advisory committee and was recommended for public release. Mention of trade names or commercial products does not constitute endorsement or recommendation by ACT for use.

Questions and comments should be directed to:

Dr. Tom Johengen
ACT Chief Scientist
CIGLR - University of Michigan
4840 S. State Street
Ann Arbor, MI 48108 USA
Email: Johengen@umich.edu

TABLE OF CONTENTS

EXECUTIVE SUMMARY

ACT conducted a performance demonstration of field-portable/-deployable assays and test kits capable of detecting Harmful Algal Bloom (HAB) toxins via immunological (i.e. antibody) and/or molecular methods. The fundamental objectives of this performance demonstration were to: (1) highlight the potential capabilities of particular field-portable assays to quantify toxins of interest including domoic acid, saxitoxins, cylindrospermopsins and microcystins; (2) verify the performance characteristics of these instruments when tested in a controlled laboratory setting, and (3) verify performance characteristics of these instruments when applied in real world monitoring applications in a diverse range of marine and freshwater coastal environments. We recognize up front, that the sampling approach used for the Technology Demonstration did not involve enough statistical power or control samples to resolve fully the reasons for differences among the manufacturer's test kit and the comparative laboratory reference analysis.

In this report we summarize the evaluation of the MBio HAB Toxin MC/CYN Gen 1 System platform for detection of the cyanotoxins microcystins and cylindrospermopsins. This field compatible technology was in a pre-commercial state of development at the time of testing. Therefore, the Demonstration goals focused more on the ease of use in field applications and relative consistency of toxin determinations compared to standardized reference methods across different natural environments (i.e. quick environmental screens versus precision quantification for regulatory decisions). Controlled laboratory tests were also conducted as part of the Demonstration to help assess measurement ranges, response to variable mixtures of toxic and non-toxic populations, and the ability of the MBio multiplex system to simultaneously conduct microcystin and cylindrospermopsin assays. Sampling and analytical replication and ranges tested were often insufficient to establish clear relationships between the MBio test kit and the laboratory reference methods. Moreover, the testing protocols established for this demonstration were not able to resolve fully why sample measurements differed or how matrix effects might have differentially affected the MBio and reference measurement approaches. We attempted to demonstrate performance in a variety of controlled laboratory conditions and a range of natural environmental conditions. The level of agreement between the MBio and reference methods differed among the natural test environments so there is likely cross-reactivity and matrix effects that should be evaluated more fully to better understand performance capabilities. Differences in agreement among the two laboratory reference methods, ELISA and LCMS-LR, were also apparent across different field-testing sites.

In summary, the MBio kit was easy to learn and simple to operate in both laboratory and field conditions. Sample lysing and analysis was easily performed per manufacturer instructions and was performed within approximately 10 and 15 minutes for the two analysis steps, respectively. While the MBio analysis operated within a broad range of temperature conditions, it was noted by the manufacturer and observed in field testing, that temperature affected the reported detection limits and range of detection. During one field test in extreme heat (> 90 ^{o}F) the MBio reader reported an error code denoting that conditions were outside of normal operational range. In that circumstance, once the readers were cooled down by shading they resumed normal operations. We commend this built in type of quality control and direct feedback to the user.

BACKGROUND AND OBJECTIVES

The Alliance for Coastal Technologies (ACT) is a NOAA- and EPA-funded component of the US Integrated Ocean Observing System (IOOS) involving a partnership of research institutions, state and regional resource managers, and private sector companies that are interested in developing, improving, and applying sensor technologies for monitoring coastal and freshwater environments. ACT was established on the premise that instrument validation of existing and emerging technologies is essential to support both coastal science and resource management. The overall goal of ACT's demonstration program is to provide industry with an opportunity to have a third-party test their instruments in both controlled laboratory settings and in diverse field applications across a range of aquatic coastal environments to aid in identifying and addressing limitations of the technology and build community awareness of these emerging technologies. It is important to note that ACT does not certify technologies or guarantee that a technology will always, or under circumstances other than those used in testing, operate at the levels verified. ACT does not seek to determine regulatory compliance; does not rank technologies or compare their performance; does not label or list technologies as acceptable or unacceptable; and does not seek to determine "best available technology" in any form.

As part of our service to the coastal community, ACT conducted a performance demonstration of field-portable/-deployable assays and test kits capable of detecting Harmful Algal Bloom (HAB) toxins via immunological (i.e. antibody) and/or molecular methods. The fundamental objectives of this performance demonstration were to: (1) highlight the potential capabilities of particular field-portable assays for on-site detection of select phycotoxins including domoic acid, saxitoxins, cylindrospermopsins and microcystins; (2) verify the performance characteristics of these instruments when tested in a controlled laboratory setting, and (3) verify performance characteristics of these instruments when applied in real world monitoring applications in a diverse range of marine and freshwater coastal environments.

INSTRUMENT TECHNOLOGY TESTED

The MBio HAB Toxin MC/CYN Gen 1 System (here after, MBio) measures toxins produced by several cyanobacterial species and measures microcystins and cylindrospermopsins simultaneously on a microarray. MBio's HAB Toxin product solution builds on the company's novel detection platform. MBio's proprietary LightDeck® technology uses a combination of planar waveguide illumination, fluorescence imaging, microarray technology, and disposable cartridge fluidics to deliver multiplexed fluorescence immunoassay results with performance comparable to much more labor-intensive and time-consuming laboratory approaches. Building on advances in plastic optics, fluidics, diode lasers, and digital cameras, MBio's LightDeck® technology enables implementation of a reader designed as a simple USB peripheral device combined with disposable sample cartridges. Elements of the MBio System are shown schematically in Figure 1. A solid-state diode laser (639 nm) is used as the illumination source. The cartridge integrates a lens into the injection molded plastic waveguide substrate, which couples and launches the laser light down the plastic substrate. The multimode waveguide generates an evanescent illumination field at the solid assay surface. By printing a spatial array of capture spots on the assay surface, the system enables multiple spot assays to be run simultaneously in every cartridge. Evanescent field illumination makes the system relatively insensitive to the solution phase components above the assay surface. This enables assays in complex sample matrices such as raw water without requiring wash steps.

The assay detection range is typically from 0.5-5.0 µg/L for microcystin and 0.7-3 µg/L for cylindrospermopsin. These limits of detection change depending on the ambient temperature. The software automatically detects the ambient temperature and reports results and limits of detection based on the temperature.

Cell lysis is performed using a 10-minute portable bead-beating method. In this method, five mLs of sample is combined with disposable beads and blended for 10 minutes, using a modified milk frother. Mechanical disruption of the algal cell walls causes lysis, releasing toxins contained intracellularly. After this cell lysis step, the lysate can be directly added to the MBio HAB Toxin MC/CYN cartridges for toxin measurement.

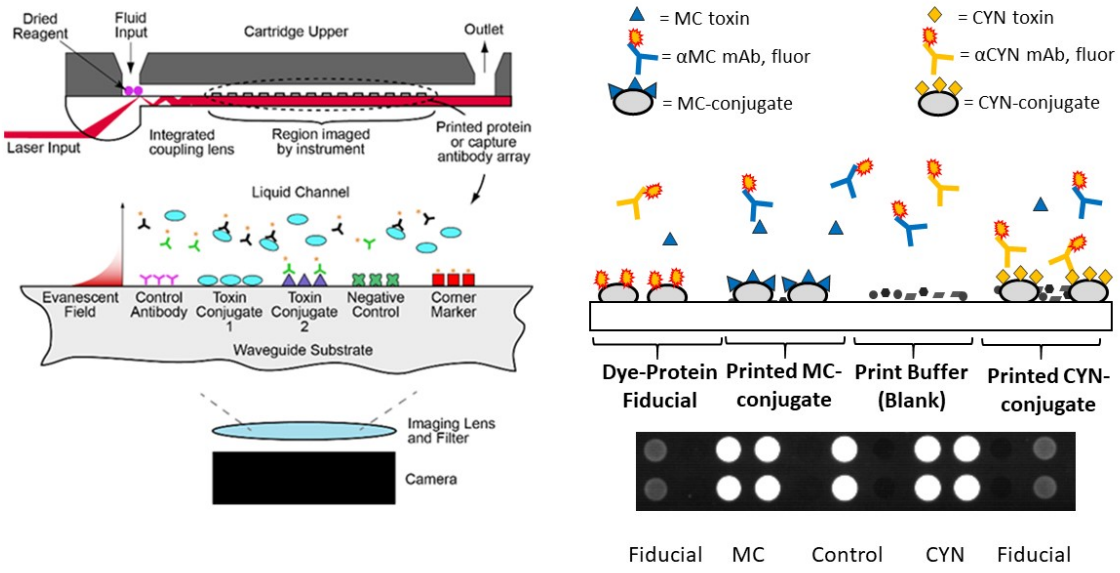


Figure 1. (*Left*) Cross-sectional schematic of the MBio LightDeck® technology. (*Upper Right*) Schematic of the array components for the duplex MC/CYN assay in the MBio cartridge. (*Bottom Right*) Representative image generated by the MBio reader.

PERFORMANCE DEMONSTRATION TEST PLAN

Rapid detection of toxin presence and concentration are vital for ensuring public safety and environmental health. Accurately and efficiently detecting and measuring harmful algal bloom (HAB) toxins in freshwater and marine systems requires specific, accurate, and time/cost-efficient technologies. Standard methods for detecting and quantifying toxins (e.g. LC-MS/MS, HPLC, mouse bioassay, receptor-binding assay) are highly accurate but tend to be time-, cost-, and labor-intensive. The time, cost and effort required to generate data from samples often means that fewer samples are analyzed and that there are significant time lags in generating those data. There are several field-portable/-deployable assays, test kits, and sensor-based approaches that detect HAB toxins via immunological (i.e. antibody) and/or molecular methods. As these approaches and instruments are incorporated into harmful algal bloom monitoring and management efforts, it is important to understand their performance. This ACT Performance Demonstration focused on a

suite of field-portable or field-deployable instruments and/or assays with the specific application of detecting HAB toxins in freshwater and marine systems.

ACT conducted two laboratory tests and four field tests as part of the toxin/HAB species instrument demonstration. One of the lab tests focused on freshwater species and associated toxins, and the second evaluated marine species and associated toxins and are described in associated reports. The field tests were chosen to represent a broad range of environmental conditions and incorporated both freshwater and marine environments. The MBio kit was only tested in freshwater environments.

Prior to laboratory testing, ACT personnel were trained on the general operations and handling of each manufacturer's specific test kit and instrumentation. Training also provided an opportunity to check operational status of instruments/kits immediately prior to the first laboratory test. A brief synopsis of the test protocols are provided below, and the complete document, *Protocols for Verifying the Performance of Algal Toxin Detection Field Sensors and Kits*, is published online at: http://www.act-us.info/evaluations.php

Laboratory Tests

A series of laboratory tests were conducted to evaluate range, accuracy, and precision of detecting freshwater HAB species and their associated toxins. The testing was performed at Bowling Green State University (BGSU) within the laboratory of Dr. Timothy Davis. The laboratory tests lasted approximately one week in duration and assessed analytical accuracy of the test instrument compared to reference sample analysis which included independent detection of toxin concentrations using USEPA adopted ELISA methods and LCMS-LR.

The freshwater lab testing took place at BGSU from July 11-15, 2018. The testing involved four different types of trials including: (1) Fortified Media Blank; (2) Analysis of common lysate from known HAB cultures; (3) Extraction and Analysis of selected toxin producing HAB cultures, and (4) a precision test with multiple instrument reads of a single fixed toxin concentration prepared from a certified standard (Abraxis MC LR certified dissolved standard, Abraxis IC #300580) dosed into a lysate produced from a culture of non-toxic *Microcystis aeruginosa* UTEX LB 2386 (https://utex.org/products/utex-lb-2386). Freshwater HAB cultures included microcystin-producer *Microcystis aeruginosa* LE3 and cylindrospermopsin-producer *Cylindrospermopsis raciborskii* CS-506. We note that LE3 is non-colonial, unlike most of the *Microcystis* occurring in natural waters.

Field Tests

A rigorous field-testing program was designed to provide a wide variety of algal toxins and toxin-producing species within various freshwater ecosystems. The selected test sites provided a range of test conditions, including ranges of cell densities, toxin concentrations, and water quality parameters such as salinity, temperature, turbidity, CDOM, and alkalinity. Each test site included sampling over multiple days and at multiple locations to provide greater variation in test conditions. For each unique environment tested, we also conducted a standard addition of a known amount of certified toxin to evaluate variability in matrix effects of the various water quality conditions and phytoplankton populations. Freshwater test sites included two locations within the Great Lakes (western Lake Erie and Sandusky Bay), as well as Pinto Lake (Watsonville, CA) and Estero Lake (Monterey, CA). The MBio kit was only tested in freshwater environments.

Reference Sample Collection and Analytical Methods

Reference samples were collected during all field and laboratory tests for direct comparison between the test instrument and independently analyzed laboratory results. All samples were processed to analyze toxin concentrations, toxin-producing genes, and phytoplankton abundance. Toxin concentrations were determined using both liquid chromatography/mass spectrometry (LCMS) and enzyme-linked immunosorbent assay (ELISA). Reference sample ELISA measurements were conducted by ACT staff at the University of Michigan using EPA Method 546 and the Abraxis kit (catalog #520011). Reference sample LCMS measurements were conducted at the lab of Dr. Raphe Kudela using state of California certified protocols. In addition, independent qPCR of targeted HAB primers and microscopic counts of targeted HAB species were conducted by ACT personnel during each lab test. Method details are described below.

Matrix effects were examined for extraction efficiency and analytical accuracy through spiked additions of certified toxin standards (Abraxis Microcystins/Nodularins (ADDA) spiking solution, Abraxis IC #300702). Results of the original ambient sample and the corresponding spiked sample were examined to assess potential challenges or variation in quantification based on phytoplankton composition and ambient water quality characteristics. At each test site a field blank was conducted utilizing toxin-free, Type 1 deionized water.

Liquid Chromatography/Mass Spectrometry

Samples for toxin analysis by LCMS-LR were collected for both whole water and the dissolved fraction. The dissolved fraction was analyzed from a 10 mL sample filtered through 0.2 µm nylon filters into amber glass vials and stored at -80ºC. The whole water fraction was analyzed from a 50 mL sample poured into amber glass bottles and stored at -80ºC. All reference samples were collected with a duplicate holdback, and samples were shipped or transported in batches on dry ice to UC Santa Cruz for analysis with the holdback remaining frozen at the local test site until results were QA'd and finalized. In the Kudela lab, samples were processed according to methods described in Mekebri et al. 2009, Kudela 2011, for microcystins, nodularin-R with the following modifications (Miller et al. 2010, Kudela et al. 2011).

Samples were received frozen and kept so at -80ºC until extraction. Sample extracts were then frozen until LCMS-LR analysis using an Agilent 6130 instrument. The established MDL based on 7x replicate analysis is 1 µg/L (on column), adjusted for sample size. Blanks were included for every 10 samples, and a standard curve was performed at the beginning/end of each set of samples. A Matrix Spike recovery was completed with each sample matrix type. The LCMS-LR used the 5-6 main microcystin congeners to analyze for both dissolved and whole water fraction of toxins. Every analytical batch included matrix additions, blanks, and standard runs. The analysis was run in full scan mode but with lower sensitivity. Microcystin results were reported as "LR" equivalents using the following coefficients indicating relative binding affinities of the MYC antibodies utilized by MBio.

EQUIV	**0.53**	**0.64**	**1**	**0.48**	**0.72**	**1**	**0.736**	**0.736**	**0.76**
Congener	**[RR]**	**[YR]**	**[LR]**	**[LA]**	**[LF]**	**[dmLR]**	**[LY]**	**[WR]**	**[NODR]**

Enzyme-Linked Immunosorbent Assays (ELISA)

Samples for microcystin toxin analysis by ELISA were collected for both whole water and the dissolved fraction. The dissolved fraction was analyzed from a 10 mL sample filtered through 0.2 µm nylon filters into amber glass vials and stored at -80ºC. The whole water fraction was analyzed from 50 mL sample poured into amber glass bottles stored at -80ºC. All reference samples were collected with a duplicate holdback which remained frozen at the local test site until results were QA'd and finalized.

ELISA analysis for microcystins was performed according to USEPA Method 546 and the Abraxis kit (catalog #520011). This procedure included a 96-well microtiter plate and competitive binding of microcystins and microcystin-protein analogues within the wells. The ELISA method employed reagent blanks, calibration standards, fortified blanks, and fortified sample matrix and duplicates. Each extract was sub-sampled into two or three wells on the plate for analytical replicates.

Quantitative Polymerase Chain Reaction (qPCR)

For quantitative polymerase chain reaction (qPCR) analysis of cyanobacteria in freshwater test sites, triplicate samples were collected on 25 mm, 2 µm pore size filters, except for west coast field samples that were collected on 0.22 µm pore size filters. Filters were stored in 2 mL polypropylene Eppendorf tubes and kept on ice until storage at -80°C. At the end of sample collection, two of the filters were extracted and analyzed and one was retained as a holdback for reanalysis if needed. Each extract was sub-sampled into two wells on the plate for analytical duplicates. ACT's qPCR analysis focused on phytoplankton toxin production genes.

The reference qPCR filters from each site/date were thawed and extracted with the QIAgen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) as this kit has been used previously to extract DNA from microbial communities during HAB events in western Lake Erie (Berry et al., 2017). Cells were disrupted by adding 100µl Buffer ATL + 30µl proteinase K, 10 sec vortex, addition of 300µl Buffer AL, 10 sec vortex, and incubation at 56ºC for 1 hour with a 10 sec vortex every 15 min. After incubation, tubes were vortexed on maximum speed for 10 min and then centrifuged for 30 sec at 20,000 g. Lysate was passed through a Qiagen QIAshredder column (20,000 g for 30 sec). qPCR detection of total 16S for total microbial quantification, and mycE/ndaF (microcystin and nodularin), cyrA (cylindrospermopsin), and sxtA (saxitoxin) for abundance of toxin biosynthetic genes in the sample was carried out by the Phytoxigene™ CyanoDTec kit according to the manufacturer's protocols. Reactions were run on a QuantaBio Q qPCR machine (https://www.quantabio.com/) with the following cycling parameters: initial denaturation step of 120 sec at 95ºC followed by 40 cycles of 10 sec at 95ºC (3ºC per second ramp rate) and 45 sec at 64ºC (1.5ºC per second ramp rate). Copy numbers per reaction were calculated by the software which compared the cycle at which the sample exceeded background fluorescence (Cq value) compared to Cq's from a full standard curve that covered five orders of magnitude ($1x10^2$ – $1x10^6$ copies per reaction; proprietary Phytoxigene™ components) generated prior to the start of the field campaign and imported for each analysis. If the Cq for the internal control (supplied in the Phytoxigene™ component) within each reaction (IAC) was greater than 1.5 cycles above 31, it was considered inhibited and the sample was diluted and re-run.

Cell Counts

Phytoplankton cell abundances were quantified for each reference sample to determine relative abundance of cyanobacteria. For the cell counts, whole water samples (20 mL) were fixed with 1 mL of acidified Lugol's for a final preservative concentration of 4% (v/v). Cell abundance of cyanobacteria was enumerated microscopically according to methods described in Brierly, et al. 2007 after concentrating as necessary by settling or gentle centrifugation (3000 rpm, 10 min).

Ancillary Measurements

In addition to reference sample analysis, site-specific conditions were recorded with a multi-parameter YSI EXO 2 sonde during each field test. The EXO2 sonde was calibrated prior to use at each site and collected water quality characterization for temperature, conductivity/salinity, turbidity, fDOM, and pigment fluorescence during reference sample collection.

Quality Management

All technical activities conducted by ACT comply with ACT's Quality Management System (QMS), which includes the policies, objectives, procedures, authority, and accountability needed to ensure quality in ACT's work processes, products, and services. The QMS provides the framework for quality assurance (QA) functions, which cover planning, implementation, and review of data collection activities and the use of data in decision-making, and quality control. The QMS also ensures that all ACT data collection and processing activities are carried out in a consistent manner, to produce data of known and documented quality that can be used with a high degree of certainty by the intended user to support specific decisions or actions regarding technology performance. ACT's QMS meets the requirements of ISO/IEC 17025:2005(E), *General requirements for the competence of testing and calibration laboratories*; the American National Standards Institute (ANSI)/American Society for Quality (ASQ) E4-2004 *Quality Systems for Environmental Data and Technology Programs*; and U.S. Environmental Protection Agency, quality standards for environmental data collection, production, and use.

RESULTS OF LABORATORY TEST

Freshwater Lab Test

The freshwater lab test occurred during July 11-15, 2018 at Bowling Green State University and utilized various mixtures of a microcystin-producing culture of *Microcystis aeruginosa* (LE3), a culture of non-toxin producing isolates of *Microcystis* (LB 2386), and a cylindrospermopsin producing culture of *Cylindrospermopsis raciborskii* (CS-506). *M. aeruginosa* LE3 and LB 2386 were grown in BG-11 medium and *C. raciborskii* CS-506 was grown in Jaworski's Medium. All cultures were maintained at 20 °C under a light intensity of 5 µmol m^{-2} s^{-1} and a 12:12 L:D cycle. It should be noted that LE3 is non-colonial, unlike most of the *Microcystis* occurring in natural waters. Toxin production was confirmed by in-house ELISA analyses prior to start of the experiments.

Common Lysate Trial

The first laboratory trial consisted of analyzing a common lysate made from the toxic LE3 *Microcystis* culture using the USEPA freeze-thaw Method 546. This test was designed to directly compare the analytical measurement accuracy of the test instrument and eliminate any difference

between reference sample analyses that might arise from the manufacturer's toxin extraction process. Lysates were created at two cell densities of the LE3 culture (approximately 36,000 cells/mL [sample BG01] and 65,000 cells/mL [sample BG02]). It should be noted these cell densities were chosen to generate nominal toxin concentrations that fell within the standard detection range of the test instrument (0.5 - 5 µg/L) and not necessarily indicative of cell densities within a natural bloom. A third lysate concentration (sample BG03) was created by a direct one-third dilution of the lysate from (BG01) to better characterize low-end detection capability (Table 1). Microscopy results of the LE3 samples, post-testing, indicated that the culture was not pure and that a significant amount of *Planktothrix* was also present. The counts on *Planktothrix* were quite variable, in part because it was not an intended target and may have been inconsistently identified during counting.

Table 1. Quantities (mLs) of culture volumes diluted into 1 liter of media and resulting cell counts for samples used to create the common lysate test samples during the BGSU freshwater laboratory testing. Sample BG03 was created by diluting BG01 lysate to one-third of the original sample concentration.

Sample ID	**mLs LE3**	**Microcystis cells/mL**	**Planktothrix cells/mL**	**Cylindrospermopsis cells/mL**	**Total cells/mL**
BG 01	1.0	19,239	16,543	-	35,782
BG 02	3.0	57,581	6,883	-	64,464

Reference sample qPCR results of the culture samples used to prepare the common lysates confirm the relative proportion of cells across the samples, however the ratio of 16S gene copies only increased by a factor of approximately 2x versus the expected increase of 3x based on mLs of culture added. Copies per liter for the 16S gene marker were about 8x higher than estimates of cyanobacteria cell densities by microscopic counts indicating additional bacterial load in these non-axenic cultures. The proportion of the potentially toxin producing strains of *Microcystis*/*Planktothrix* containing the mcyE gene marker was approximately 1% of the cell density estimates by Microscopy (Figure 2). These unexpected large differences between cell counts and qPCR measurements indicate that these ancillary measurements should be regarded as qualitative and indicative of relative density differences across the different sample preparations.

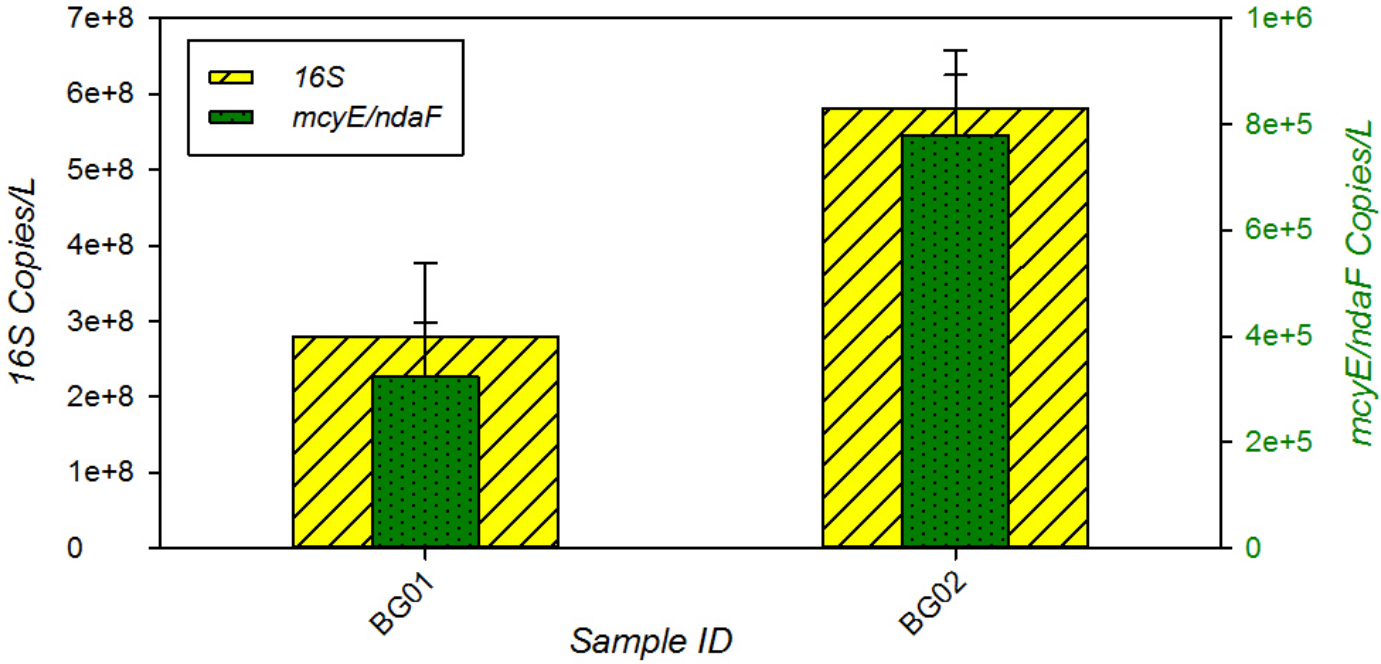


Figure 2. Estimates of cell density in gene copies per milliliter derived from the QuantaBio Q qPCR using the Phytoxigene CyanoDTec kit. Results are given for 16S total cyanobacteria copies per milliliter (left axis, yellow bars) and the mycE/ndaF (microcystin + nodularin) toxin gene copies per milliliter (right axis, green bars). Note: BG 03 is a dilution of the lysate from BG01, therefore no qPCR results were completed for this sample. Error bars represent one standard deviation of the average for the two sample replicates and the two analytical replicates of a given sample extract (n = 4).

Microcystin (MC) toxin concentration determined by MBio for the common lysate test are shown against reference sample estimations based on laboratory ELISA and LCMS-LR (Figure 3). Only a single sample was produced for each concentration. MBio results were in close agreement at the two lowest concentrations (BG01 and BG03) but slightly under-predicted the concentration of BG02 (6.2 µg/L) compared to both ELISA (7.7 µg/L) and LCMS-LR (9.1 µg/L). The reported MC value for BG02 estimated by MBio was the average of four readings (5.7, 7.0, 6.2, 6.0) the first two of which were reported out directly by the reader and the latter two were estimated after sample dilution of 1:1 with media blank after initial reporting of above detection.

Although it was a very limited range and number of comparisons, cross plots of the comparative concentration estimates for the MBio relative to ELISA and LCMS-LR concentrations are given (Figure 4). In general, the MBio response agreed with reference estimates across the range tested and with less than a 60% difference for the worst agreement at the higher concentrations.

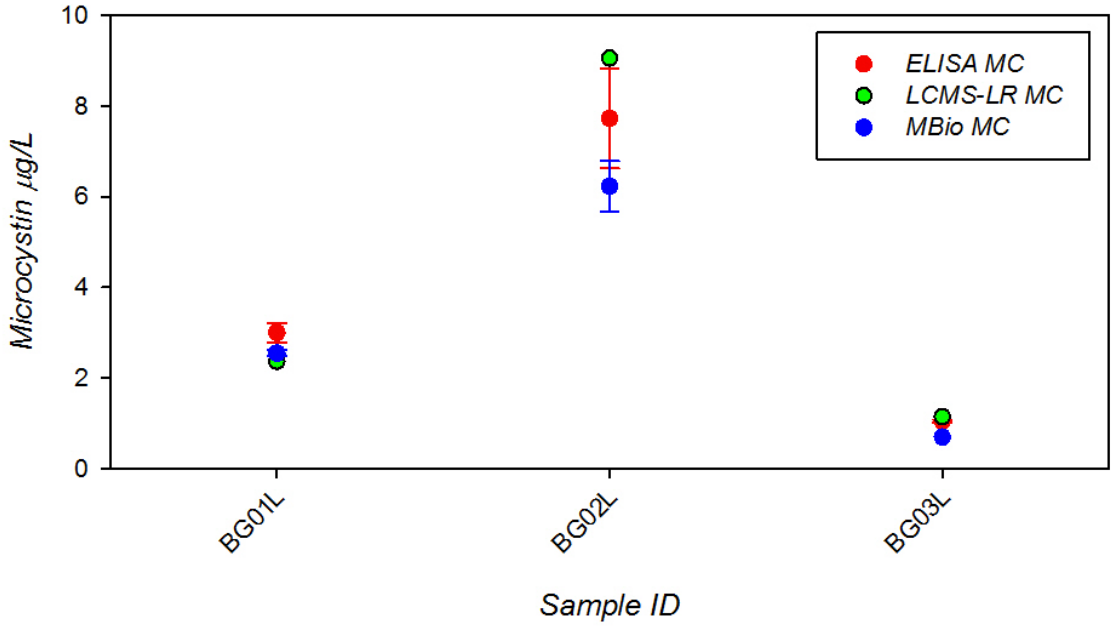


Figure 3. Comparison of microcystin estimates of samples analyzed by MBio (blue), ELISA (red), and LCMS-LR (green) from the BGSU lab common lysate trial. Error bars are one standard deviation of the analytical replicates (n=2) for MBio (except n=4 for BG03) and ELISA. LCMS-LR has no error bars as it is a single value. BG01 concentration of LE3 is 1:100, BG02 concentration is 3:100, BG03 concentration is 1/3 the concentration of BG01.

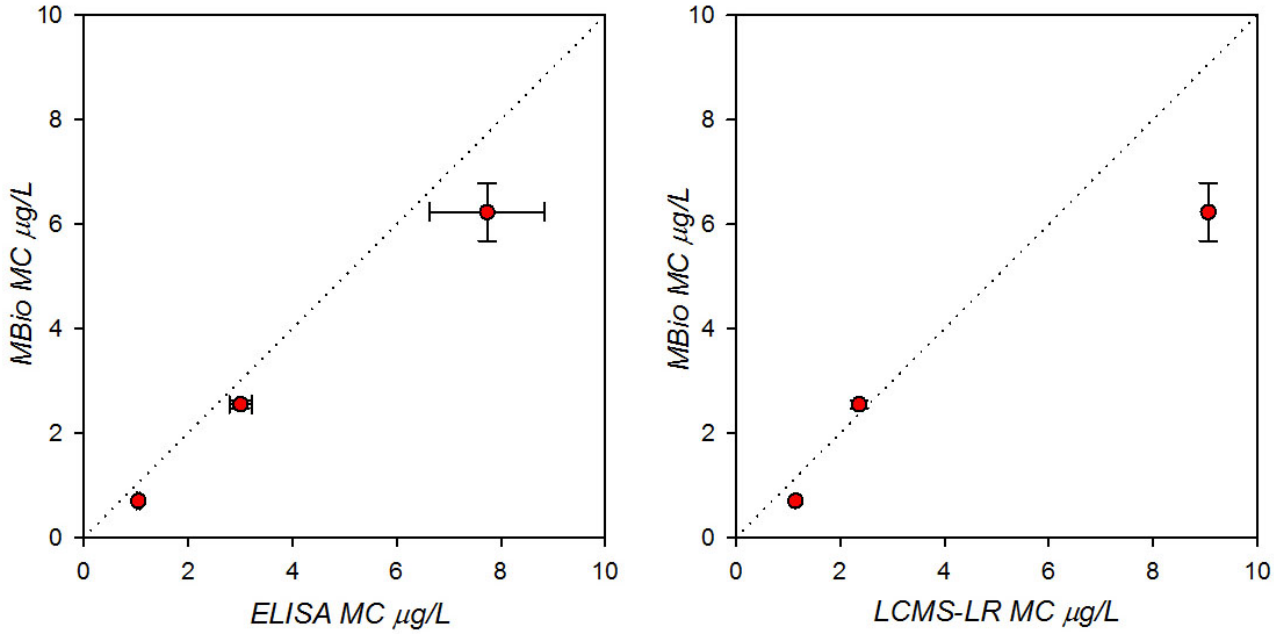


Figure 4. A cross plot of the MBio measurements compared to reference ELISA (left) and LCMS-LR (right) for the common lysate laboratory trial. Error bars are one standard deviation of analytical replicates (n=2) for MBio and ELISA data, LCMS-LR data has no error bars as it was a single value.

Mixed Species Trial

A second lab test was conducted using mixtures of two toxin producers, LE3 *Microcystis* and CS-506 *Cylindrospermopsis* at four different concentration ratios (BG06, BG18, BG19 and BG20; Table 2). A sample of each culture separately (BG04 was LE3 only and BG05 was CS-506 only) was also tested. It should be noted that the mixed species test occurred on two different days with samples BG04 – BG06 on July 12 and samples BG18 - BG20 on July 15 so that a greater range of mixtures could be tested. Microscopic counts of resulting cell densities (Table 2) are somewhat variable and make it difficult to evaluate how much change occurred in the stock cultures over the three-day interval but the test samples represented the targeted range of toxin values of between 0 to 6 µg/L for both microcystin and cylindrospermopsin. Cell densities as a function of the amount of culture added were highly variable and again there was notable contamination of *Planktothrix* in the LE3 culture.

Table 2. Quantities (mLs) of culture of LE3 and CS-506 used to create samples for the mixed species trial, and the computed cell density of the generated samples based on microscopic counts.

Sample ID	mLs LE3	mLs CS-506	Microcystis cells/mL	Planktothrix cells/mL	Cylindrospermopsis cells/mL	Total cells/mL
BG 04	1.0	-	24,677	38,057	-	62,735
BG 05	-	1.0	-	-	19,270	19,270
BG 06	1.0	1.0	24,769	5,690	19,239	49,697
BG 18	3.0	1.2	20,427	160	6,444	27,031
BG 19	1.5	2.4	14,761	11,836	25,500	52,097
BG 20	0.7	4.8	9,854	9,568	41,915	61,337

qPCR results for 16S, mcyE/ndaF, and cyrA gene markers for these generated reference samples are presented in figure 5. Like the cell count data, there was considerable variability in the number of gene copies of each marker relative to the proportions of the culture used to make the sample mixtures. However, the results generally confirm the presence and relative abundances of the targeted species and their associated toxins.

A comparison of MBio results for microcystin concentration against the reference ELISA and LCMS-LR measurements is presented in figure 6. For five of the six test samples the reference LCMS-LR values were substantially higher than the ELISA values. The MBio measurements tend to agree more closely with the ELISA reference data, with the exception of sample BG19 when the LCMS-LR value was lower than expected relative to the other sample mixtures. The cross plots of MBio and reference sample analysis show a more consistent agreement to the ELISA measurements than to LCMS-LR and had a significant linear regression (slope = 0.85, $R^2 = 0.95$, p=0.005; Figure 7 left panel).

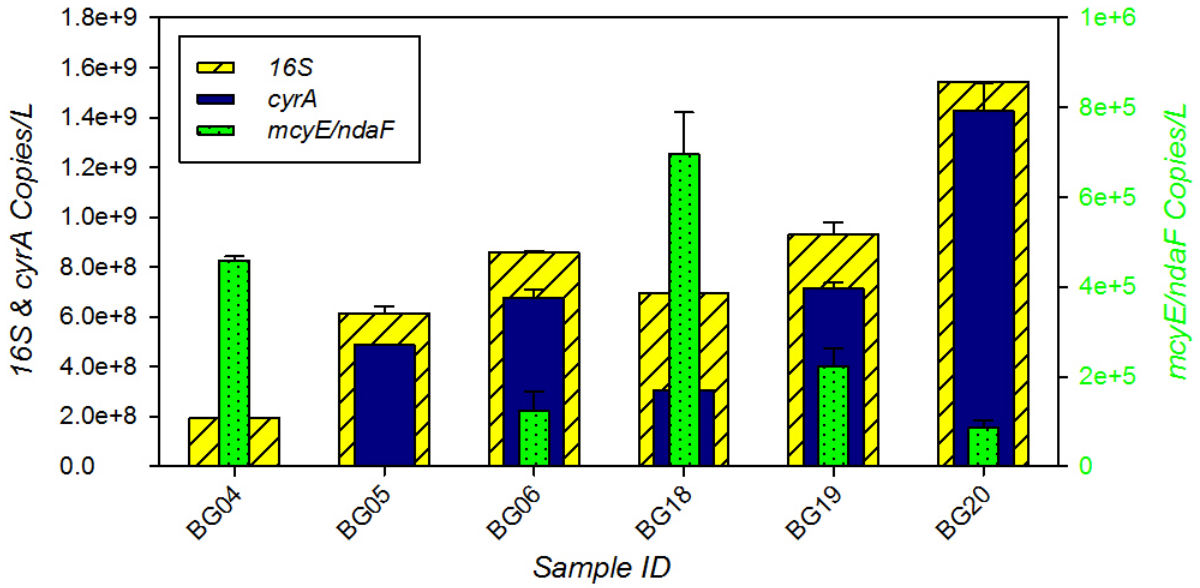


Figure 5. qPCR results for the mixed species lab test. Estimates of target gene markers in copies per liter derived from the QuantaBio Q qPCR using the Phytoxigene CyanoDTec kit. Results are given for 16S (yellow bars), mycE/ndaF (right axis, green bars), and cyrA toxin gene copies per milliliter (blue bars). Error bars represent one standard deviation of two sample replicates each with two analytical reps (n = 4).

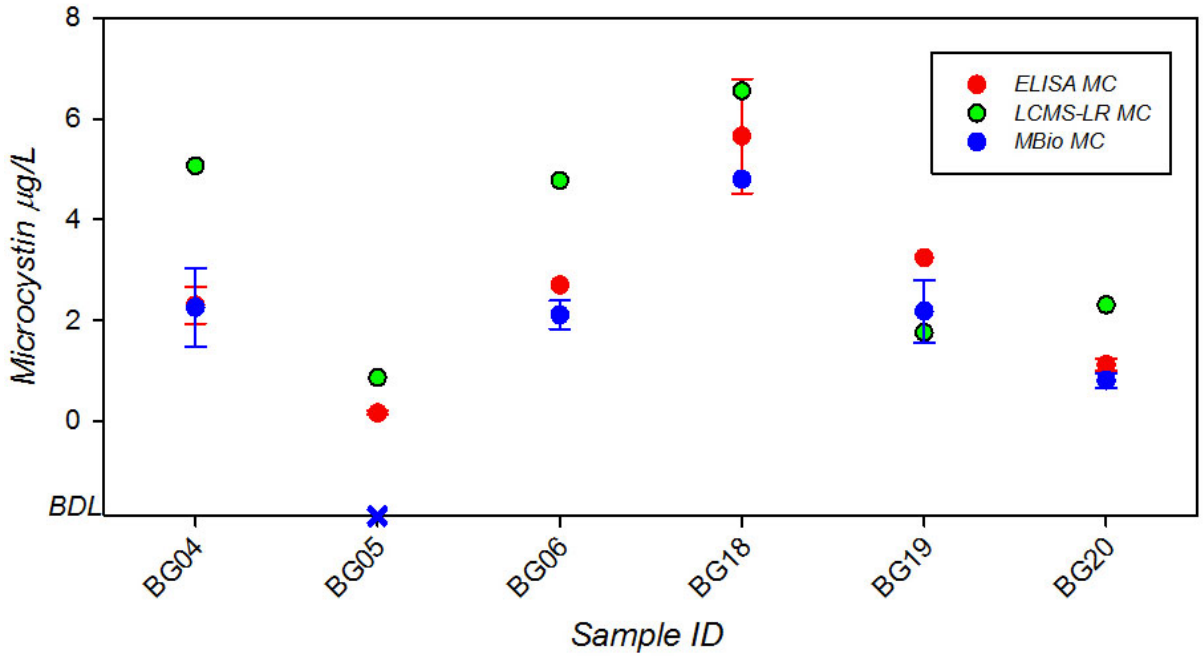


Figure 6. Comparison of MC toxin concentration measurements for the BGSU mixed species trial using *Microcystis* (LE3) and *Cylindrospermopsis* (CS-506). Results are plotted for the MBio test system (blue), reference ELISA (red) and LCMS-LR (green) microcystin data. Error bars are one standard deviation (n = 2) for MBio and ELISA, LCMS-LR have no error bars as there was a single value. BG05 resulted in a value below detection (BDL<0.5 µg/L) for the MBio. We note the *Microcystis* culture was contaminated with *Planktothrix*.

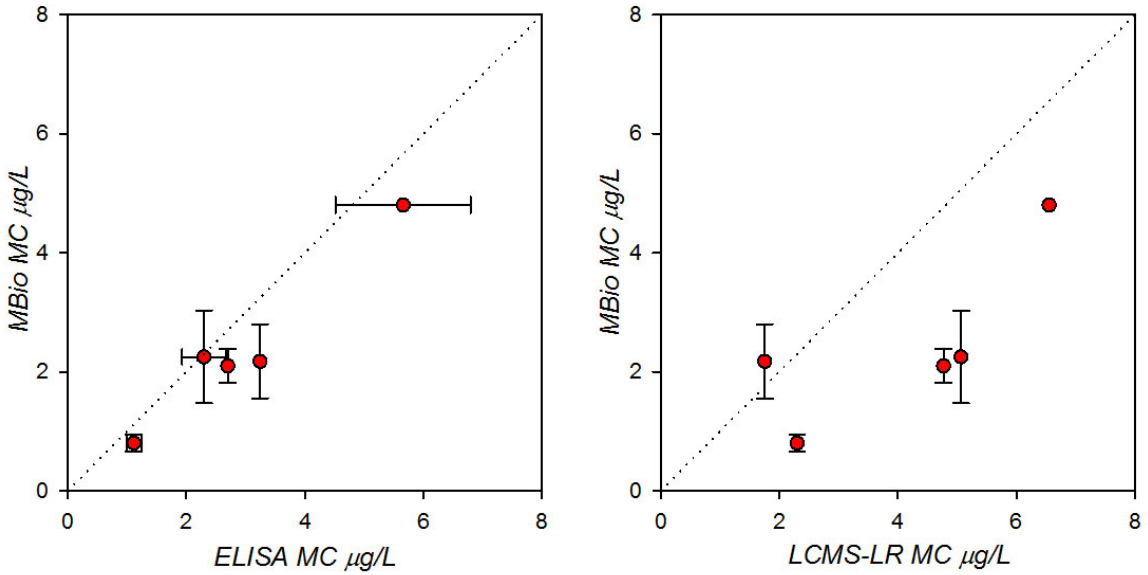


Figure 7. Cross plots for the MBio measured microcystin compared to reference ELISA (left) and LCMS-LR (right) for the laboratory mixed species trial. Error bars are one standard deviation (n=2), LCMS-LR data has no error bars as there was a single value. MBio BDL data (<0.5 µg/L) is not included.

MBio measurements of the toxin CYN were compared against reference sample qPCR measurements of the cyrA gene target since no corresponding ELISA or LCMS data were generated for CYN (Figure 8). The MBio estimations tracked the cyrA cell copy estimates reasonably well over the tested range, but exhibited a small false positive reading in the BG04 sample with no *Cylindrospermopsis.*

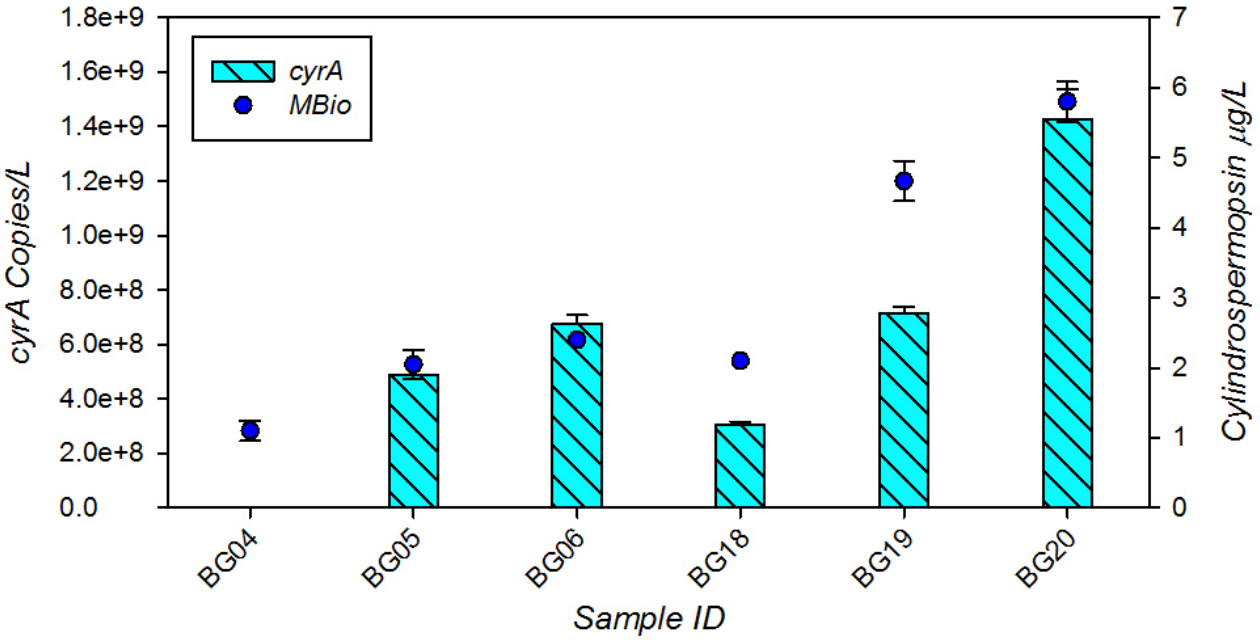


Figure 8. Comparison of results for the QuantaBio Q qPCR cyrA toxin gene target (bars) and MBio test system cylindrospermopsin (circles) for the BGSU mixed species trial using *Microcystis* (LE3) and *Cylindrospermopsis* (CS-506). Error bars are one standard deviation of n = 2 for MBio and n = 4 for QuantaBio Q.

A cross plot of MBio CYN and qPCR data confirms the general linear agreement and non-zero intercept (Figure 9). The linear regression was significant ($p=0.01$; $R^2=0.83$) but it is acknowledged that there are few data points and less correspondence at the low end of the range.

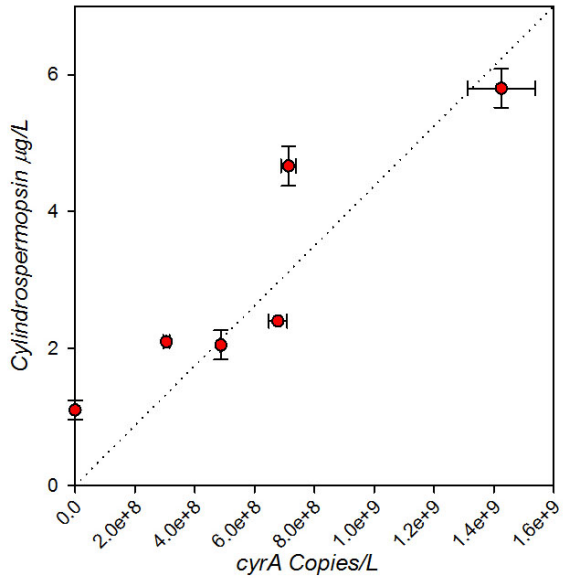


Figure 9. BGSU mixed species response plot of MBio cylindrospermopsin estimates compared to reference Quanta Bio Q qPCR data for the cyrA gene target. Below detection data not included in the graph.

Range Trial

The laboratory range trial consisted of mixtures of both toxic *Microcystis* (LE3) and non-toxic *Microcystis* (LB 2386). Mixtures of the two cultures were generated at six different concentration ratios intended to cover a 16-fold toxin concentration range, along with a media only negative control (Table 3). Sample BG10 and BG14 were independently created sample duplicates to examine consistency through all stages of sample preparation, processing, and analysis.

Table 3. Quantities (mLs) of toxic and non-toxic cultures added to generate test samples for the BGSU lab range trial with corresponding microscopy based cell counts. Samples BG10 and BG14 are duplicates in terms of the culture mixtures but were produced independently. Sample BG13 was a media blank with no culture added. We note the *Microcystis* LE3 culture was contaminated with *Planktothrix*.

Sample ID	**mLs LE3**	**mLs LB 2386**	***Microcystis* (LE3 & LB 2386) cells/mL**	***Planktothrix* cells/mL**	**Total cells/mL**
BG 13	0.0	0.0	0.0	0.0	Media Only
BG 07	1.0	15	56,971	28,181	85,152
BG 08	2.0	14	51,221	13,488	64,709
BG 09	4.0	12	49,698	10,682	60,380
BG 10	8.0	8.0	45,039	10,638	55,677
BG 14	8.0	8.0	50,472	3,377	53,849
BG 11	12	4.0	45,851	7,007	52,859
BG 12	16	0.0	44,054	6,718	50,772

Results for qPCR gene marker copies for the range trial reference sample are shown in figure 10. A total of 16 mLs of culture were added to each sample, with the proportion of toxic and non-toxic strains of *Microcystis* varied as shown in Table 3. The 16S gene marker copies (Figure 10) were relatively consistent across the mixtures (a factor of two), but somewhat inconsistent with the microscopic cell count data (Table 3). The mcyE/ndaF gene marker copies generally followed the dosing pattern of LE3 with the exception of samples BG08 and BG10, which were larger than expected.

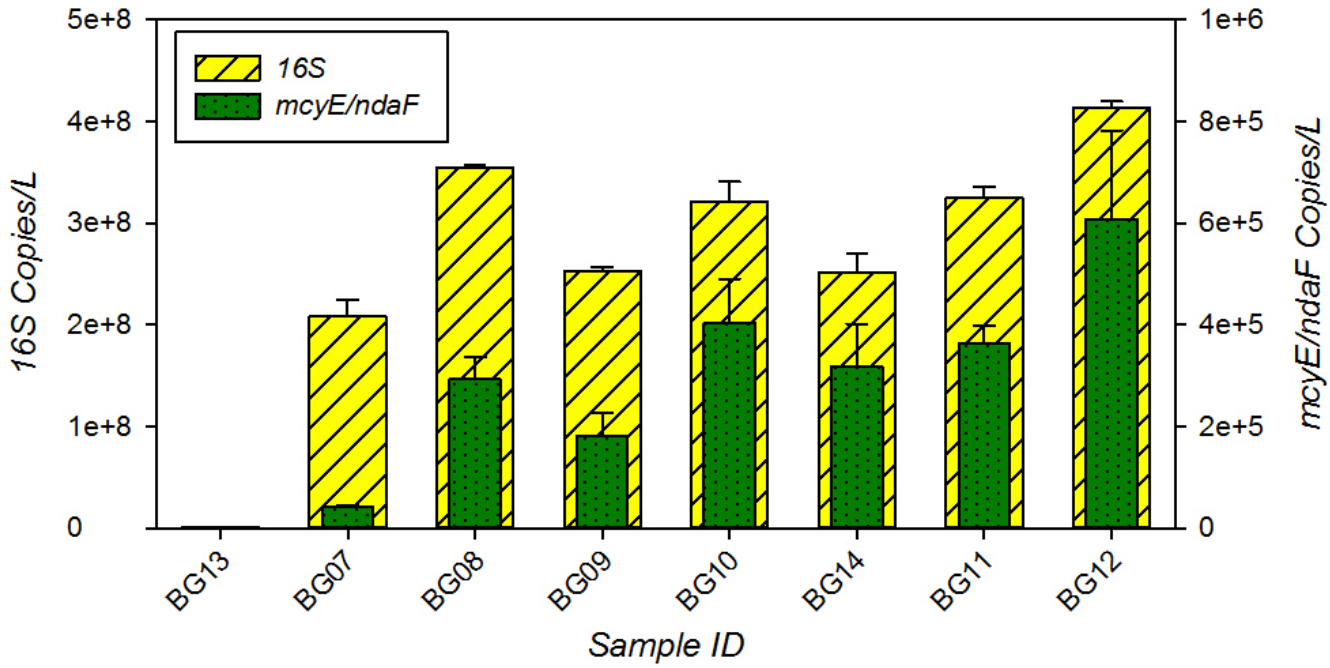


Figure 10. QuantaBio Q qPCR results for range trial. Samples BG10 and BG14 are duplicates in terms of the culture mixtures but were made independently. Sample BG13 was a media blank with no culture added. Error bars represent one standard deviation of the average for the two sample replicates and the two analytical replicates of a given sample extract (n = 4).

The MBio measured MC concentrations track the ELISA and LCMS-LR measurements reasonable well at all but the highest concentration tested. We note that toxin measurement at this highest concentration required a 3-fold dilution and recognize that matrix dilution may affect the various analyses differently. We also note that the reference method ELISA and LCMS-LR measurements themselves also diverged significantly at higher MC concentrations (Figure 11). With sample BG12 omitted the slopes and R-squares were 0.38 and 0.97 regressed against ELISA and 0.54 and 0.97 regressed against LCMS-LR. Unlike most previous tests, the MBio estimations agreed better with LCMS-LR estimations versus ELISA at all but the highest concentration. Cross plots using the entire test range are shown in figure 12 and the linear regressions were still significant for ELISA comparisons (slope = 1.49, R^2 = 0.76), as well as, for LCMS-LR comparisons (slope = 2.13, R^2 = 0.76).

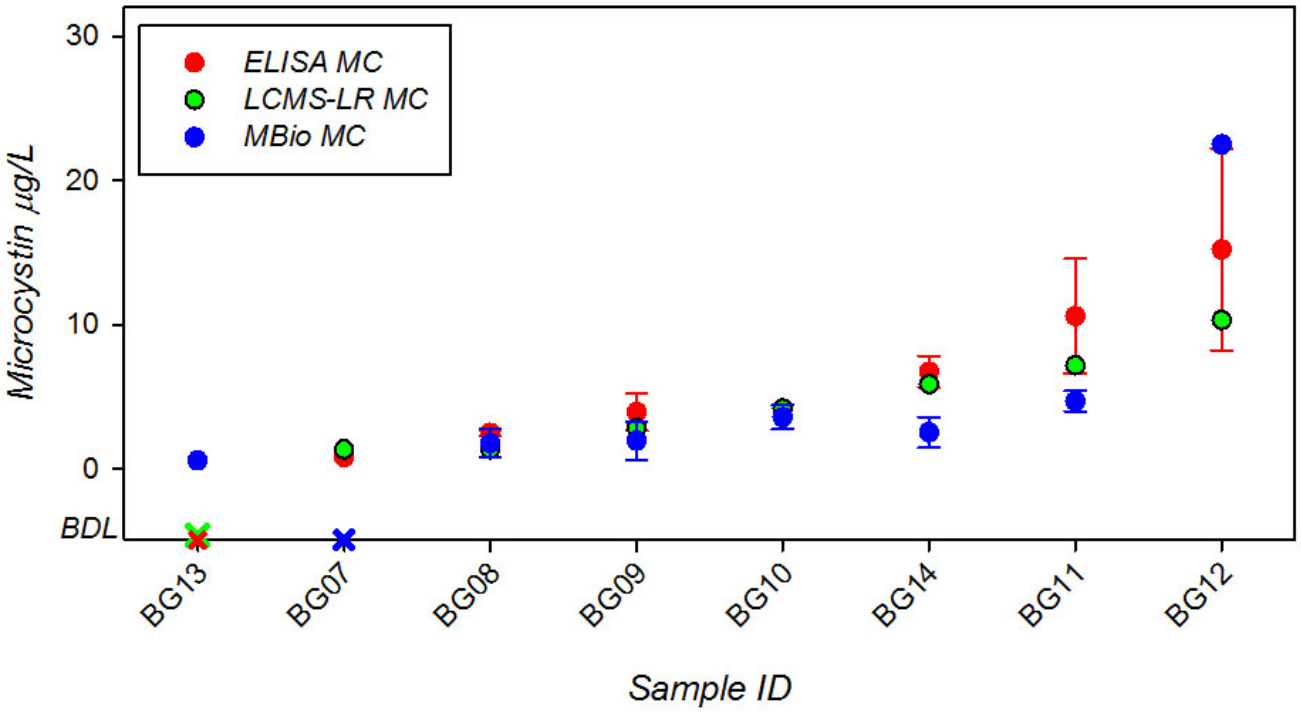


Figure 11. Comparison of microcystin estimates of samples analyzed by MBio (blue), ELISA (red), and LCMS-LR (green) from the BGSU lab range trial. Error bars are one standard deviation (n=2) for MBio and ELISA, LCMS-LR has no error bars as it is a single value. Values plotted on the X-axis represent values reported as below detection. Samples BG10 and BG14 are sample replicates made individually to check for consistency in both sample preparation and analysis.

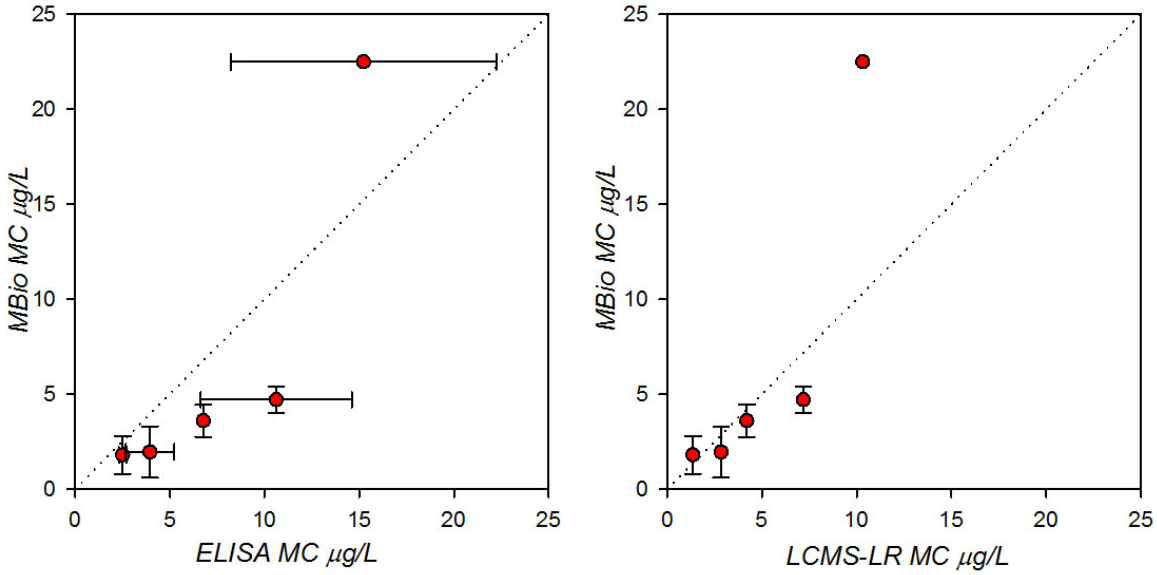


Figure 12. A cross plot of MBio measurements compared to reference ELISA and LCMS-LR microcystin measurements for the laboratory range trial. Error bars represent one standard deviation (n = 2) for MBio and ELISA, LCMS-LR have no error bars as there was a single value.

Precision Trial

A precision test was conducted that involved multiple instruments reads of a single fixed toxin concentration prepared from a certified standard (Abraxis MC LR certified dissolved standard, Abraxis IC #300580) dosed into a lysate produced from a culture of non-toxic LB 2386 to provide a background matrix. The targeted toxin concentration by dilution was 1.50 µg/L. The reference sample ELISA measurements was 1.9 µg/L with a standard deviation of 0.53. The reference sample LCMS-LR measurements was 3.97 µg/L (single measurement only). The MBio measurements ranged from 1.1 to 2.3 µg/L, with an average of 1.5 µg/L and a standard deviation of 0.35. It is unclear why the LCMS-LR estimation was so much higher and there were no remaining back-up samples to re-analyze.

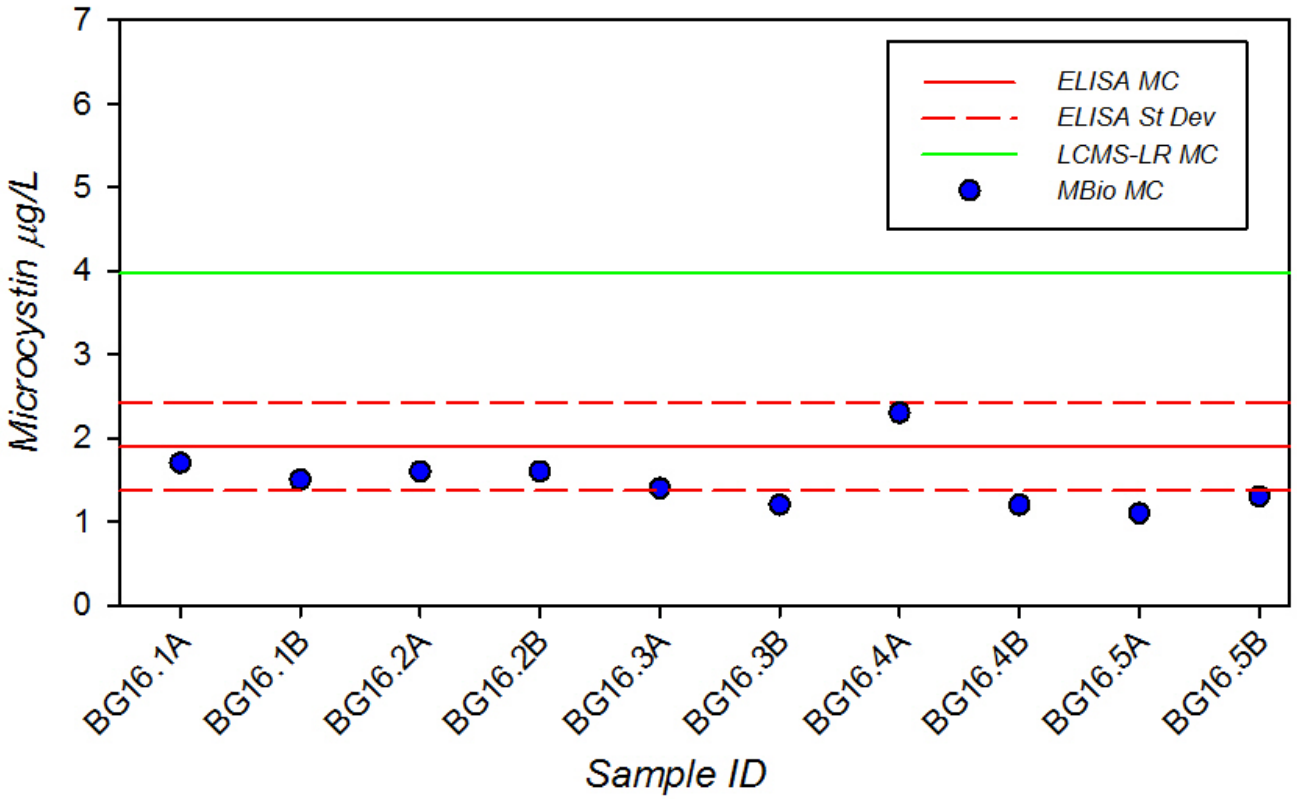


Figure 13. Results of the precision trial laboratory test using repeated measurement of a diluted certified MC standard to a targeted concentration of 1.5 µg/L, the ELISA estimated the concentration at 1.9 ±0.53. Five measurements each were made on two MBio readers denoted as A and B.

Certified Standard Trial

The final component of the freshwater laboratory testing was a single sample analysis (BG17) of an Abraxis MC LR certified dissolved standard (Abraxis IC #300580) diluted in MilliQ water to a nominal concentration of 1.2 µg/L. The MBio measurement was 1.2 ±0.0 compared to an ELISA result of 1.29 ± 0.01 µg/L and an LCMS-LR result of 3.49 µg/L.

RESULTS OF FIELD TESTS

Four freshwater field tests were conducted as part of the MBio performance demonstration including Western Lake Erie and Sandusky Bay in the Great Lakes, and Pinto Lake near Watsonville, CA and El Estero Lake near Monterey, CA. The Western Lake Erie and Sandusky Bay locations were both sampled on two different occasions to capture a greater dynamic range in HAB toxin conditions. Three different locations were sampled on each occasion. During each sampling trip a fourth sample was generated that was either an independent field replicate or a spiked addition of an aliquot from one of the existing field samples.

Lake Erie and Sandusky Bay

Samples were collected from three coastal monitoring stations in Western Lake Erie on July 24, 2018 and August 30, 2018. Two of the stations were located in Maumee Bay (WE 06, WE 09), and the third station (WE 02) was located approximately 12 km off shore from the mouth of the Maumee River near the Toledo Harbor Lighthouse (Figure 14 left panel). Western Lake Erie routinely experiences extensive blooms of *Microcystis*, a microcystin producing algae, from July through September. Samples were collected using 2 L Van Dorn samplers deployed from the NOAA R/V4108 and processed dockside within approximately two hours of collection.

Sandusky Bay is located in the southeastern corner of Lake Erie's western basin. The bay is shallow (mean depth ~ 2 meters) and well mixed with annual microcystin producing *Planktothrix agardhii*-dominated algal blooms occurring from May - October. Water was collected from three dockside stations along the southern shore of Sandusky Bay on August 14 and August 22, 2018 (Figure 14 right panel). At each station, 8 L of whole surface water was collected using a horizontal 2 L Van Dorn sampler. Two homogeneous samples were created by splitting each van dorn equally across acid-washed and triple DI rinsed 4 L collection bottles. A YSI EXO2 sonde was used to collect physicochemical data at each site. Samples were processed within approximately two hours of collection.

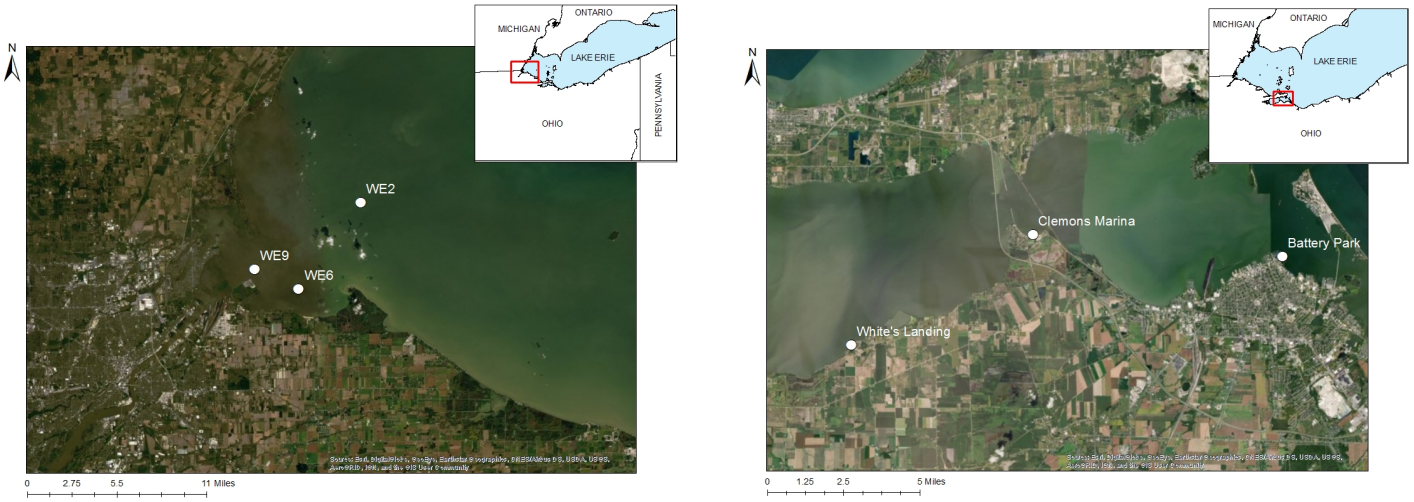


Figure 14. Western Lake Erie and Sandusky Bay sample stations. Western Lake Erie sampling stations (left) for WE2, WE6, and WE9. Sandusky Bay sampling locations (right) for Whites Landing, Clemons Marina and Battery Park.

Each Great Lakes field sample was processed and analyzed for both total and dissolved microcystin concentration. The whole water (total) toxin measurements for MBio were produced

using the manufacturer's provided field lysing system followed by immediate measurement on the reader. Results for total and dissolved toxin measurements are broken out and described separately. Ancillary cell counts and extracted chlorophyll, were generated for each of the Great Lakes samples to evaluate differences in phytoplankton composition and relative sample matrix conditions (Table 4). Reference sample qPCR results for 16S and mcyE/ndaF gene markers are presented in figure 15. It is interesting to note that despite the significantly higher amount of phytoplankton biomass and 16S copies in Sandusky Bay, western Lake Erie samples contained as many or more MC toxin producing cells as noted by the copies of mcyE/ndaF.

Table 4. Ancillary cell counts and extracted chlorophyll for the Great Lakes field tests. Samples on 7/24 and 8/30 were collected in western Lake Erie and samples on 8/14 and 8/22 were collected from Sandusky Bay. Samples GL02 and GL03 were independently collected field duplicates, samples GL06 and GL07 were field duplicates. Sample GL11 was made by spiking GL10 with 0.5 µg/L of a dissolved MC standard. Sample GL16 was made by spiking GL13 with 0.5 µg/L of a dissolved MC standard (Abraxis Microcystins/Nodularins (ADDA) spiking solution, Abraxis IC #300702).

Date	Sample ID	Location	Microcystis Cells/mL	Planktothrix Cells/mL	Extracted Chlorophyll a (µg/L)
7/24/18	GL 01	WE2	34,807	0	9.4*
	GL 02	WE6	65,654	0	34.4*
	GL 03	WE6 (**field dup**)	70,453	2,133	no data
	GL 04	WE9	29,574	13,644	34.5*
8/14/18	GL 05	White's Landing	0	432,427	146
	GL 06	Clemons Marina	0	281,906	121
	GL 07	Clemons Marina (**field dup**)	0	399,980	123
	GL 08	Battery Park	0	243,804	73.9
8/22/18	GL 09	White's Landing	0	283,677	115
	GL 10	Clemons Marina	0	292,131	100
	GL 11	Clemons Marina **(spiked)**	0	295,672	no data
	GL 12	Battery Park	0	159,432	73.5
8/30/18	GL 13	WE2	61,237	0	29.7
	GL 14	WE6	89,022	0	49.7
	GL 15	WE9	52,880	1,469	36.9
	GL 16	WE2 **(spiked)**	67,254	609	no data

*Results from samples taken on 7-23-18 by NOAA GLERL at this site on the day prior to testing because chlorophyll samples were not processed from the day of collection.

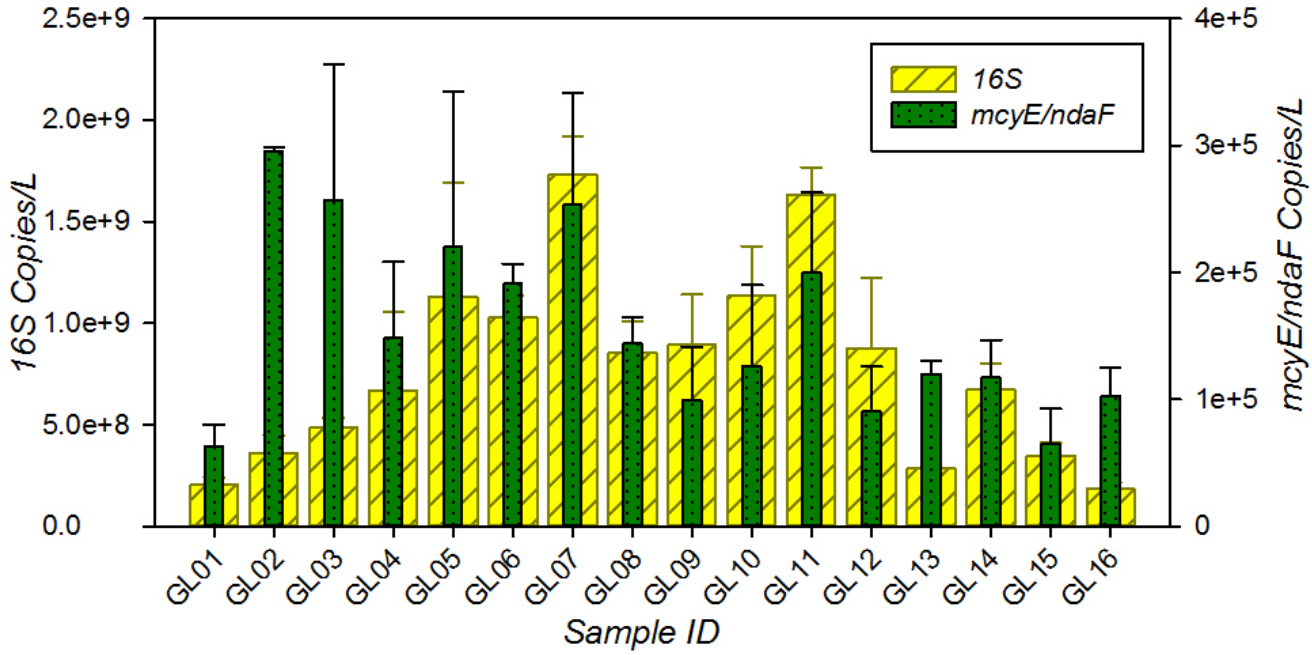


Figure 15. QuantaBio Q qPCR results for Great Lakes field testing in western Lake Erie (GL01-GL04 and GL13-GL16) and Sandusky Bay (GL05-GL12). GL02 & GL03 and GL06 & GL07 are independently collected field sample duplicates. Sample GL11 is a spiked addition of sample GL10 and sample GL16 is a spiked addition of GL13. Both spikes were made by the addition of 5mLs of Abraxis Microcystins/Nodularins (ADDA) spiking solution (Abraxis IC #300702) into a 500 mL sample for an addition of 0.5 µg/L MC. Error bars represent one standard deviation of the average of two analytical reps from each of two filter replicates (n=4).

A comparison of **total** MC toxin measurements for MBio and corresponding reference sample ELISA and LCMS-LR measured MC for the western Lake Erie and Sandusky Bay field trials is presented in figure 16. The comparison includes the following quality assurance samples: samples GL02 and GL03 were field duplicates; samples GL06 and GL07 were field duplicates; sample GL11 was made by spiking GL10 with 0.5 µg/L of a dissolved MC standard; and sample GL16 was made by spiking GL13 with 0.5 µg/L of a dissolved MC standard. Specific results for these samples are presented below in Table 5 of the QA/QC section. There were distinct differences in the comparability of toxin measurements between ELISA and LCMS-LR for the two different Great Lakes' environments. For western Lake Erie, dominated by *Microcystis*, there was a much closer agreement between the two reference sample analyses. For Sandusky Bay samples, the ELISA MC measurements were 4 to 8 times higher than the LCMS-LR measurements. It is likely that some of the MC toxin congeners produced by *Planktothrix* were not resolved by the adopted LCMS-LR analysis.

The MBio toxin measurements were in close agreement with both ELISA and LCMS-LR measurements for the western Lake Erie samples. However, the relative relationship of the measurements changed from week 1 to week 4. During the 7/24 sampling event, the MBio estimations were all above the reference sample measurements. Conversely, during the 8/30 sampling event the MBio estimations were mostly lower than the reference sample measurements. It is not possible to determine whether these differences were related to sample matrix effects,

phytoplankton composition, or changes in instrument performance. Although the differences were not large, the shift in relative response caused significant variation in the direct cross plot comparison of the MBio and reference sample data as shown in figure 17.

For Sandusky Bay samples, the MBio toxin measurements always fell between the ELISA and LCMS-LR measurements suggesting they may have been less influenced by matrix effects or cross-reactivities. Again, there was substantial variation in the direct comparison between MBio and reference sample ELISA and LCMS-LR as noted in the one-to-one cross plots (Figure 18). Given the high degree of analytical variability and the variability in response across the two sampling events, linear regressions between MBio and reference method estimations were not statistically significant.

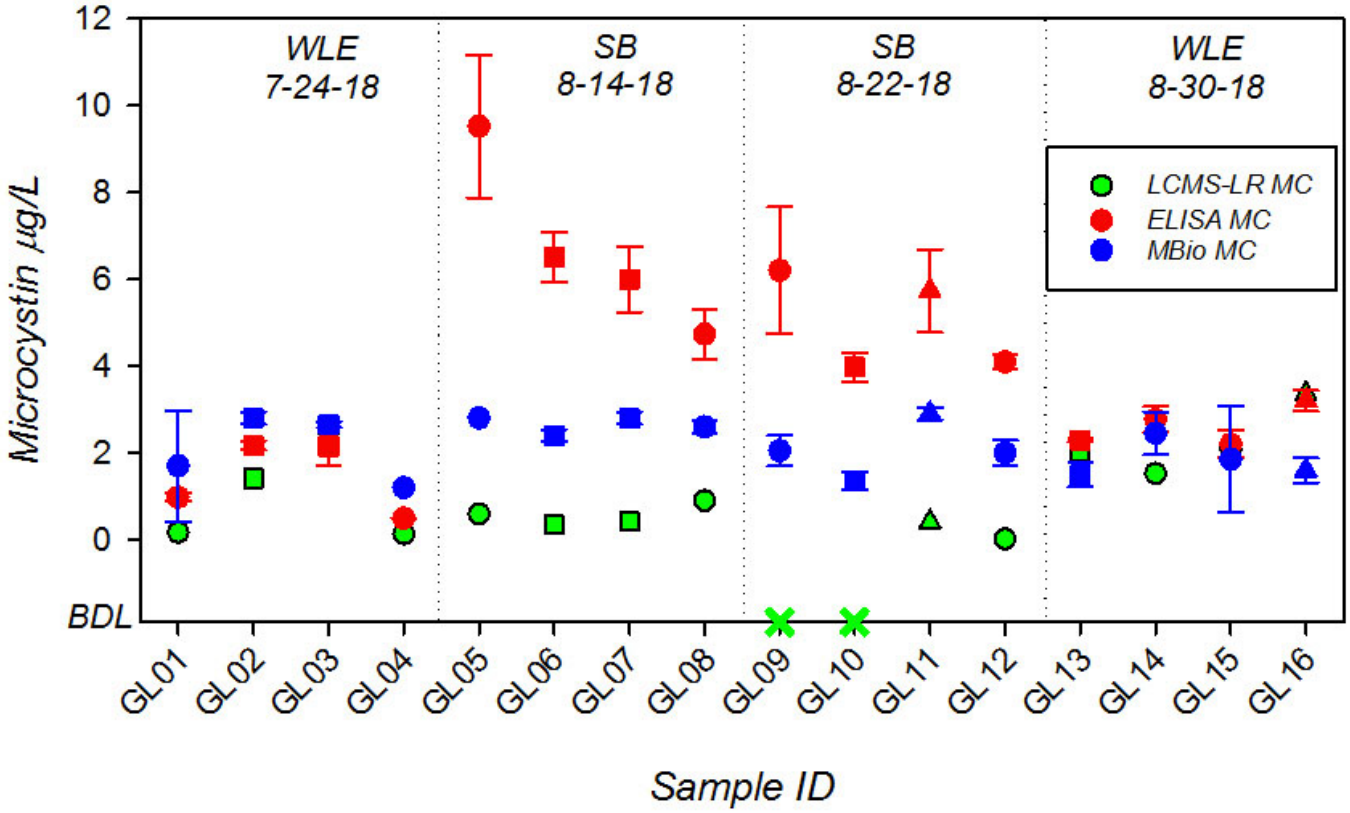


Figure 16. Great Lakes Field comparison of MBio (blue), reference ELISA (red) and LCMS-LR (green) **total** microcystin data. Samples GL 01-04 and GL 13-16 were taken in western Lake Erie, samples GL 05-12 were taken in Sandusky Bay. Square symbols represent duplicate samples, triangular symbols represent a spiked sample duplicate, and crosses represent samples reported below detection (LCMS-LR BDL varies for each congener, all <0.5 µg/L). Error bars represent one standard deviation for MBio and ELISA (n=2), LCMS-LR has no error bars as there was only one value.

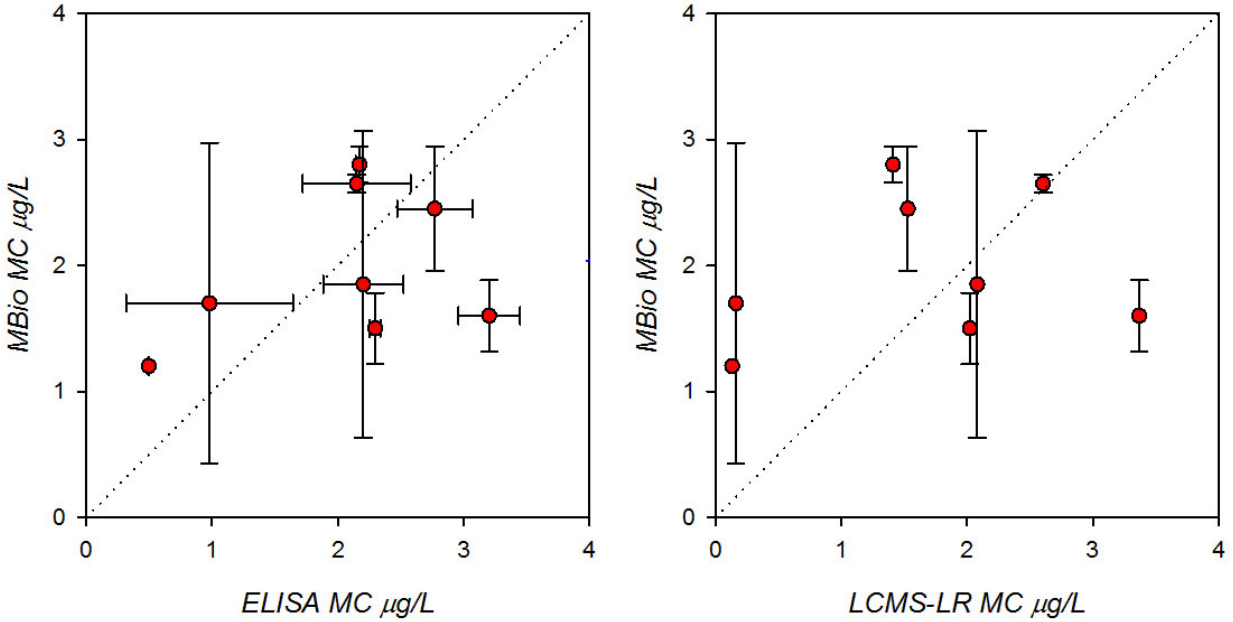


Figure 17. Western Lake Erie field response plot of MBio compared to the ELISA (left) and LCMS-LR (right) whole water reference data. Error bars represent one standard deviation for MBio and ELISA (n=2), LCMS-LR has no error bars as there was only one value. Data below detection is not included.

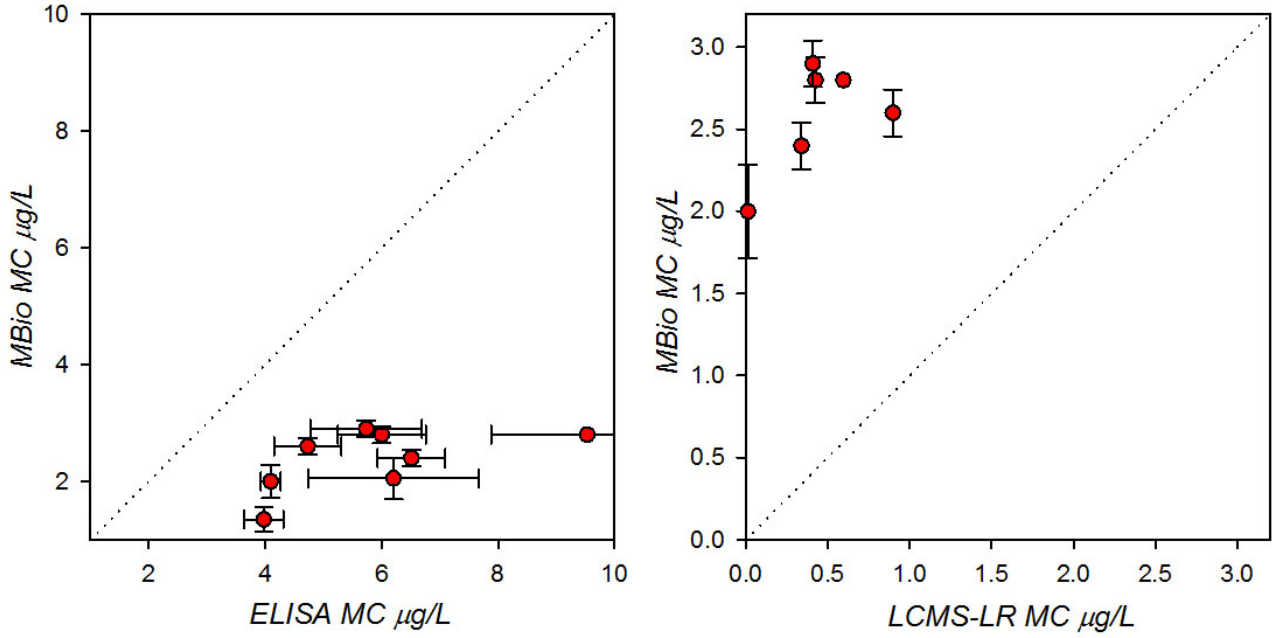


Figure 18. Sandusky Bay field response plot of MBio compared to the ELISA (left) and LCMS-LR (right) whole water reference data. Error bars represent one standard deviation for MBio and ELISA (n=2), LCMS-LR has no error bars as there was only one value. Data below detection is not included.

A comparison of **dissolved** MC toxin measurements for the MBio instrument and corresponding reference sample ELISA and LCMS-LR measured MC for the western Lake Erie and Sandusky Bay field trials is presented in figure 19. In general, the dissolved fraction represented only 10 to 20 % of the whole-water microcystin fraction and in 10 out of 16 cases was at or below the stated detection limit for MBio and LCMS-LR. This finding was typical for the active growth stage of cyanobacterial blooms with the bulk of toxin associated with particulate fraction. There were again substantial differences between the reference samples measured by ELISA and LCMS-LR for Sandusky Bay. ELISA estimations were again significantly higher than LCMS-LR for the dissolved fractions and three of the six independent samples were non-detects by LCMS-LR. There were too few observations and limited range to generate meaningful statistical relationship but one-to-one cross plots for quantified sample measurements are shown by region in figures 20 and 21.

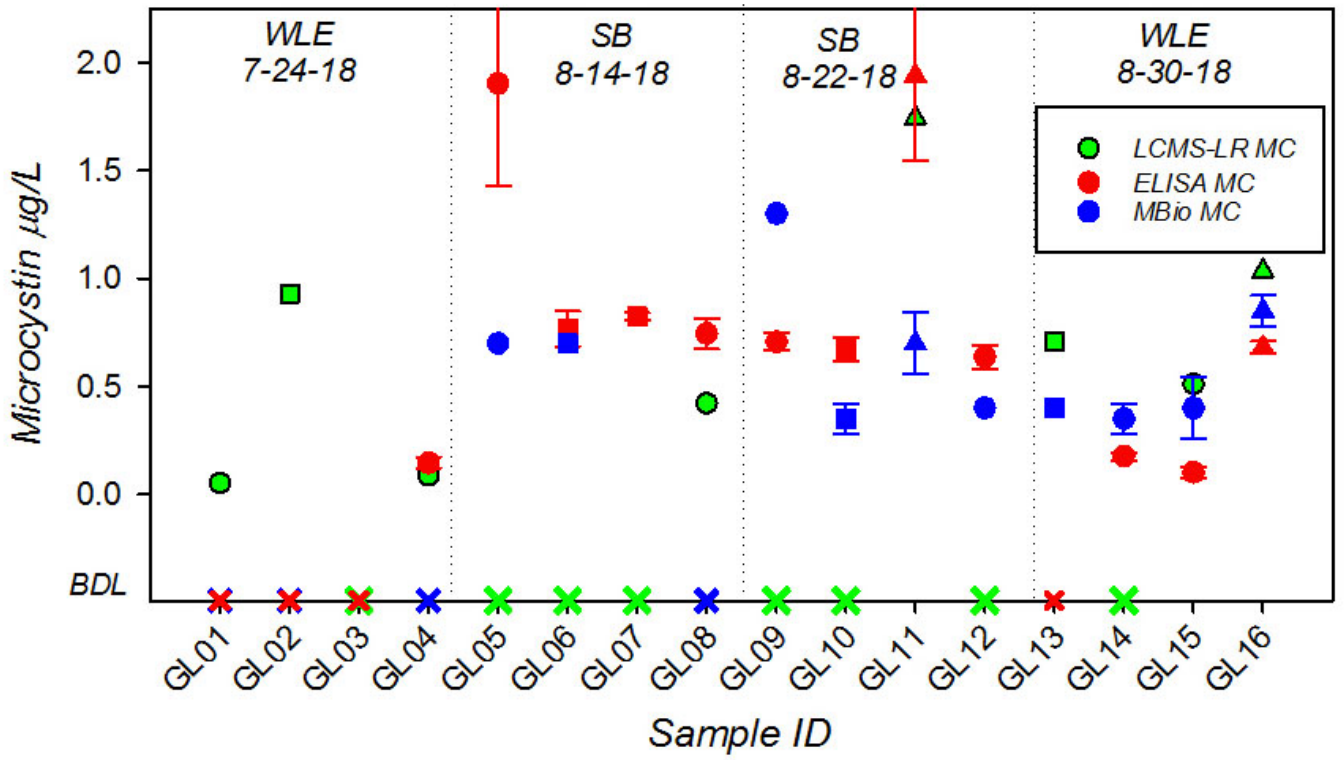


Figure 19. Great Lakes Field comparison of MBio (blue), reference ELISA (red) and LCMS-LR (green) **dissolved** microcystin data. Samples GL 01-04 and GL 13- 16 were taken in western Lake Erie, samples GL 05-12 were taken in Sandusky Bay. Square symbols represent duplicate samples, triangular symbols represent a spiked sample duplicate, and crosses represent samples reported below detection (MBio BDL < 0.5 µg/L, ELISA <0.1 µg/L, LCMS-LR BDL varies by congener, all <0.5 µg/L). Error bars denote one standard deviation for MBio and ELISA (n=2), LCMS-LR has no error bars as it was a single value.

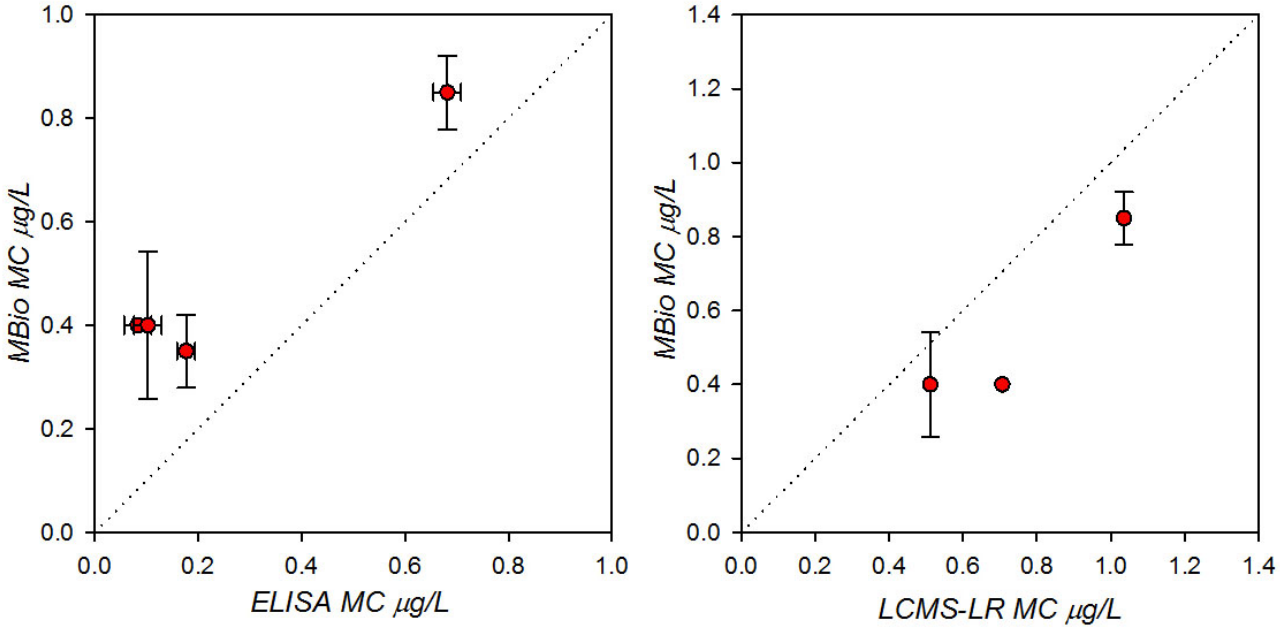


Figure 20. Western Lake Erie field response plot of MBio compared to the ELISA (left) and LCMS-LR (right) dissolved toxin reference data. Error bars represent one standard deviation for MBio and ELISA (n=2), LCMS-LR has no error bars as there is only one value. Below detection data is not included in the plot.

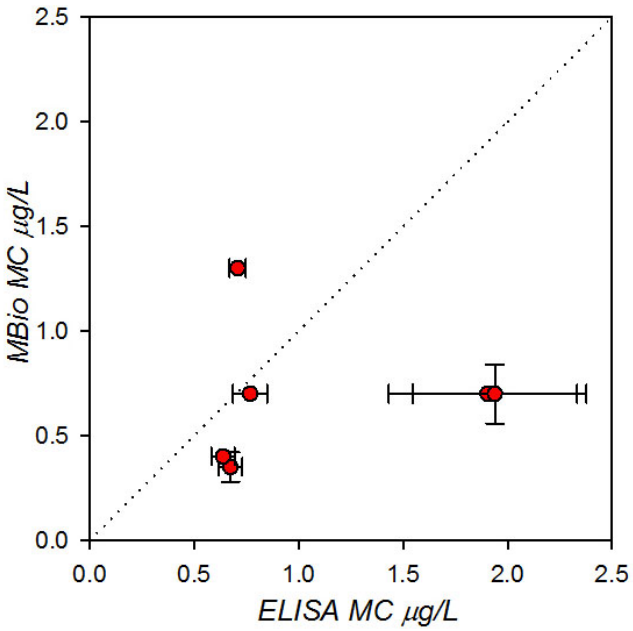


Figure 21. Sandusky Bay field response plot of MBio compared to the ELISA dissolved toxin reference data. Error bars represent one standard deviation for MBio (n=2) and ELISA (n=2). Below detection data is not included in the plot. LCMS-LR response plot is not included due to the large number of BDL values for LCMS-LR.

Pinto Lake and El Estero Lake, California

Microcystis aeruginosa, *Cylindrospermopsis* and *Planktothrix* were not observed at detectable levels in Pinto Lake (36.9554° N, 121.7715° W; Watsonville CA) leading up to and throughout the testing period (as monitored by weekly routine sampling conducted by the Kudela lab at UCSC). Nevertheless, we collected samples from Pinto Lake on two occasions (sample ML14 on September 17th; samples ML16-ML19 on September 18th) and added one additional field sample from a small local lake in Monterey, CA, El Estero Lake (36.5989° N, 121.8856° W; sample ML15 on September 17th). Samples ML18 and ML19 are independently collected field duplicates from Pinto Lake to evaluate representativeness of sample collection along with variability in sample processing and analysis. Surface samples were collected via a plastic bucket and composited into one carboy to homogenize before processing. Samples were analyzed for both total and dissolved toxin concentrations. We note that for the field testing on September 18 that air temperature exceeded 90 ºF, and the MBio readers detected that the temperature was too high to report an accurate result. According to operational design, the MBio reader reported a temperature error indicating that the sample could not be analyzed. Once the readers were cooled down by shading they resumed analysis. It is unclear to what extent, if any, the high heat conditions may have impacted performance but it has been noted by the manufacturer that elevated temperatures increases the limit of detection output.

qPCR analysis of the reference samples indicated that the total cyanobacterial densities, (estimated by copies of the 16S gene marker) were 1-2 orders of magnitude lower than seen in the Lake Erie field trials. Furthermore, copies of mcyE/ndaF gene markers were all below quantification (Figure 22).

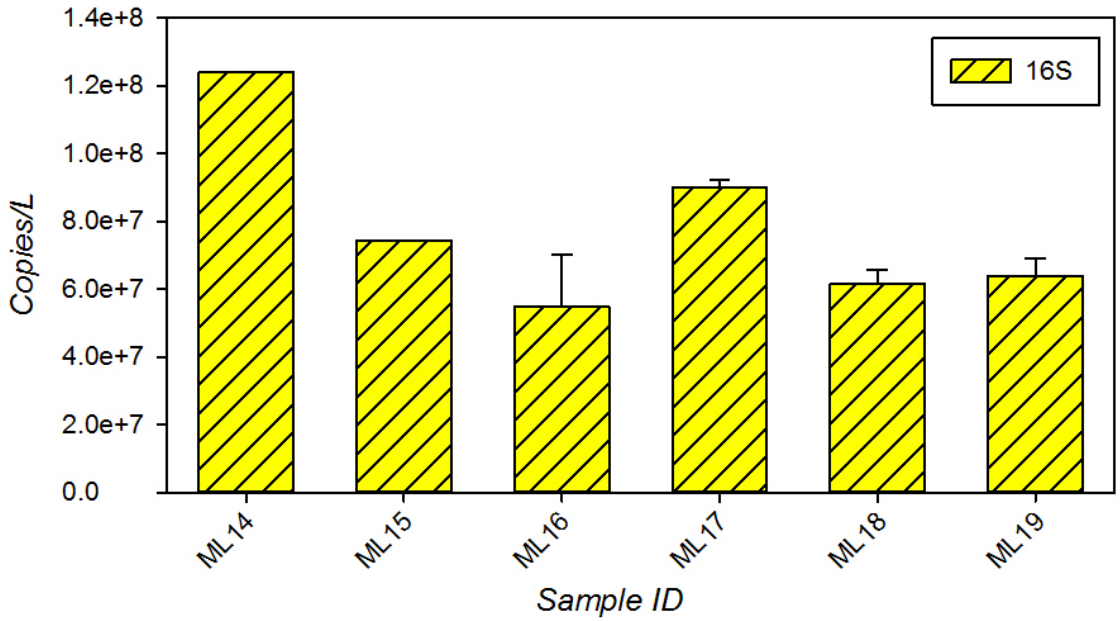


Figure 22. QuantaBio Q qPCR results for Pinto Lake (ML14, 16-19) and El Estero Lake (ML 15) reference samples. Copies of the mcyE/ndaF gene markers were all below quantification (< 45 copies per reaction) so no data are presented. Error bars are one standard deviation of two filters and two analytical replicates (n = 4).

Despite no detectable mcyE/ndaF gene targets, small levels of microcystin were detected by MBio and occasionally by the reference methods for (Figures 23 and 24). For ELISA results, measurements fell between 0.06 – 0.09 µg/L and are all reported as BDL based on the established methodological detection limit of 0.1 µg/L. For LCMS-LR there were two positive reads for whole water samples (ML14 and ML17) and one positive read for dissolved MC (ML14). The MBio estimated whole water MC concentrations near 1 µg/L for samples ML14, ML15, ML18 and ML19 and non-detect for ML16 and ML17. For dissolved microcystin samples, the MBio estimated concentrations ranged from 0.3 to 1.6 µg/L, whereas the only measureable detection for the reference samples was 0.4 µg/L by LCMS-LR for sample ML14 (Figures 23 and 24).

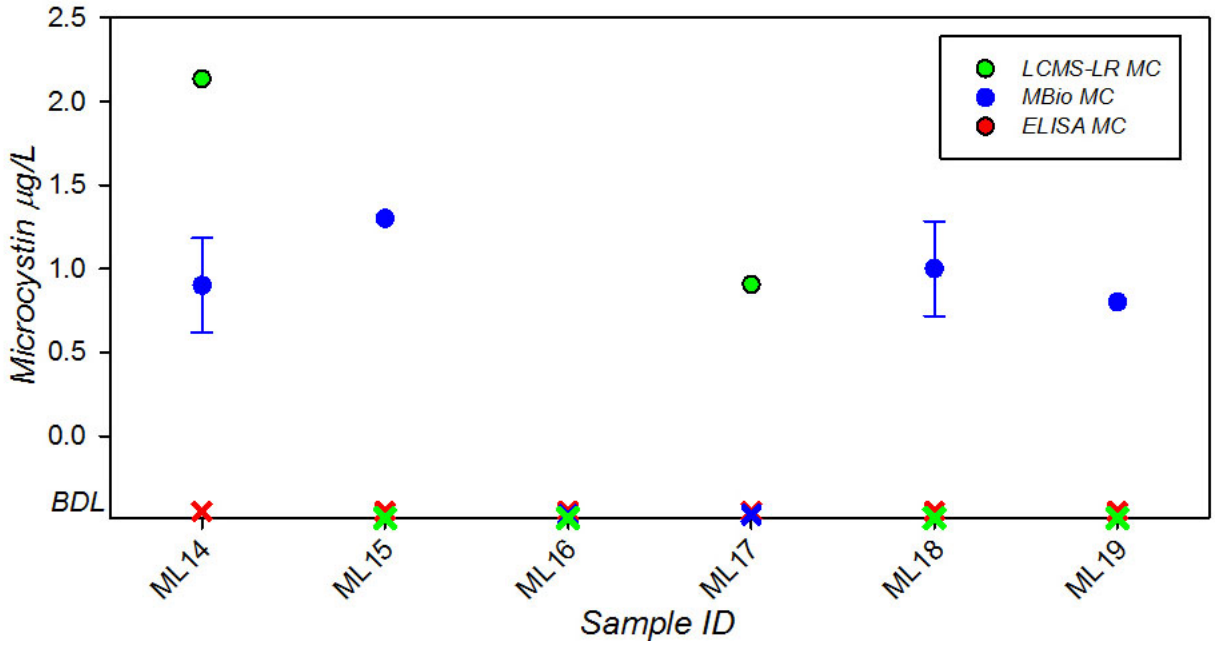


Figure 23. Pinto and El Estero Lakes (ML 15) comparison of MBio (blue), reference ELISA (red) and LCMS-LR (green) **whole** microcystin data from MLML field samples. Error bars denote one standard deviation for MBio and ELISA (n=2), LCMS-LR has no error bars as it was a single value. MBio BDL < 0.5 µg/L, ELISA <0.1 µg/L, LCMS-LR BDL varies by congener, all <0.5 µg/L. Samples ML18 and ML 19 are independently collected field duplicates.

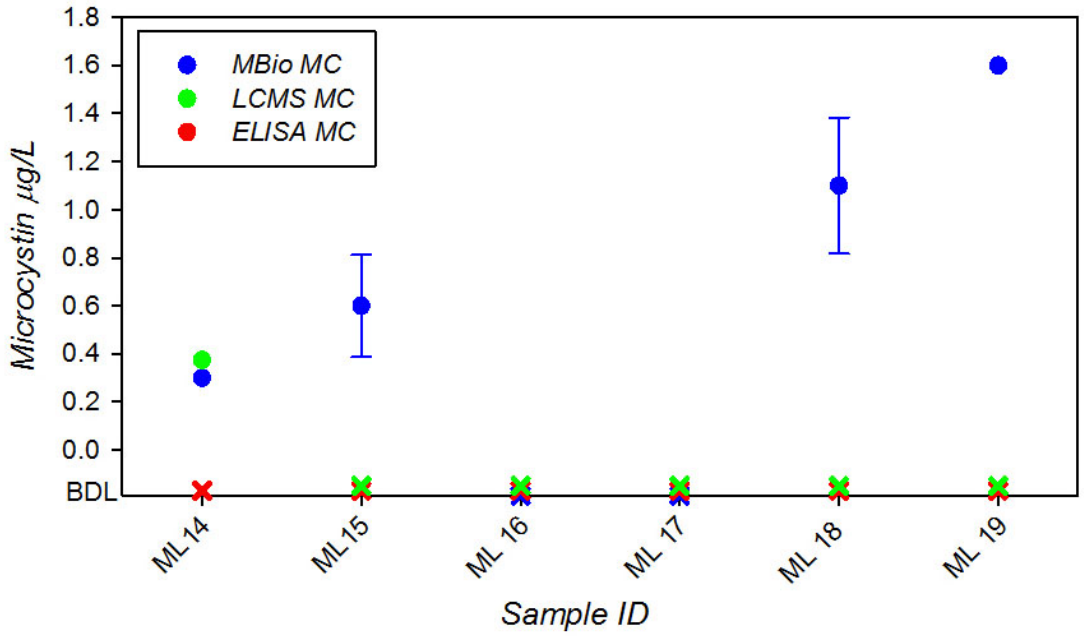


Figure 24. **Dissolved** microcystin determined in the field at Pinto (ML 14, ML 16-19) and El Estero (ML15) Lakes. Comparison of MBio (blue), reference ELISA (red) and LCMS-LR (green) dissolved microcystin data. Error bars denote one standard deviation for MBio and ELISA (n=2), LCMS-LR has no error bars as it was a single value. Crosses represent data below detection (MBio BDL < 0.5 µg/L, ELISA BDL <0.1 µg/L, LCMS-LR BDL varies by congener, all <0.5 µg/L).

QUALITY ASSURANCE AND QUALITY CONTROL

All technology evaluations conducted by ACT comply with its Quality Management System (QMS), which includes the policies, objectives, procedures, authority, and accountability needed to ensure quality in work processes, products, and services. A QMS provides the framework for quality assurance (QA) functions, which cover planning, implementation, and review of data collection activities and the use of data in decision making, and quality control. The QMS also ensures that all data collection and processing activities are carried out in a consistent manner, to produce data of known and documented quality that can be used with a high degree of certainty by the intended user to support specific decisions or actions regarding technology performance. ACT's QMS meets U.S. Environmental Protection Agency quality standards for environmental data collection, production, and use, and the requirements of ISO/IEC 17025:2017, *General requirements for the competence of testing and calibration laboratories* and the National Environmental Laboratory Accreditation Conference (NELAC) Institute (TNI) Standard FSMO-V1, *General requirements for field sampling and measurement organizations*, which is modeled after ISO/IEC 17025.

An effective assessment program is an integral part of a quality system. Technical audits help to ensure that the approved Test Protocols and applicable standard operating procedures (SOPs) are being followed, and that the resulting data are sufficient and adequate for their intended use. High quality data and effective data quality assessment are required for accurately evaluating the performance of a technology and provide confidence that the collected data are properly documented and defensible.

The ACT Quality Assurance (QA) Manager independently conducted Technical Systems Audits (TSA) of the laboratory test at Bowling Green State University on July 8-13, 2018; and field

tests in Long Island Sound during May 6-8, 2018; Pinto Lake, CA, September 18, 2018; and Monterey Bay, September 20, 2018; and a data quality review of the reference data sets from all tests.

Quality Control Sample Analysis

As part of the sample analysis quality control evaluation two media only negative controls were run as part of the Laboratory testing (see Results, Freshwater Lab Test, Range Trial). During the Lab testing one set of duplicate samples was generated during the Range Trial and comparative results are shown in Table 5. For the field testing, duplicated field reference samples were collected once each from western Lake Erie, Sandusky Bay, and Pinto Lake. Comparative results of the field duplicates are shown in Table 5. Agreement was generally better for the ELISA measurements than for LCMS-LR measurements. Lastly, one analyte spike (using dissolved MC standard) was conducted on one reference sample each from western Lake Erie and Sandusky Bay (see Table 5). The targeted spike by known addition was 0.5 µg/L of dissolved Microcystins/Nodularins (ADDA) using spiking solution MCT-LR (Abraxis IC #300702). Recoveries for whole sample analyses were considerably higher than for dissolved sample analyses or the expected amount.

Table 5. Results of independent field duplicates and spike recoveries for freshwater lab and field samples.

Sample ID	Sample Type	Whole Water ELISA µg/L	Whole Water LCMS-LR µg/L	Dissolved Fraction ELISA µg/L	Dissolved Fraction LCMS-LR µg/L	qPCR 16S Copies/L	qPCR mycE/ndaF Copies/L
BG 10	Reference	no data*	4.21	1.84	1.66	321,415,820	403,684
BG 14	Duplicate	6.76	5.88	1.80	1.64	251,576,883	317,412
Mean St. Dev		6.76 -	5.04 1.18	1.82 0.03	1.65 0.02	286,496,352 49,383,586	360,548 61,003
Coeff. Var.		-	23.4	1.64	1.03	17	17
GL 02	Reference	2.17	1.41	0.10	0.93	361,276,191	295,703
GL 03	Duplicate	2.15	2.60	BDL	BDL	483,915,580	257,261
Mean St. Dev		2.16 0.01	2.01 0.84	0.08 0.02	0.47 0.66	422,595,885 86,719,143	276,482 27,183
Coeff. Var		0.68	42.0	23.2	141.4	20.5	9.83
GL 06	Reference	6.51	0.34	0.77	BDL	1,026,398,267	191,243
GL 07	Duplicate	6.00	0.42	0.82	BDL	1,729,754,469	253,126
Mean St. Dev		6.25 0.36	0.38 0.06	0.80 0.04	BDL BDL	1,378,076,368 497,347,940	222,184 43,758
Coeff. Var.		5.75	15.74	5.04	-	36	20

GL 10	Reference	3.98	BDL	0.67	BDL	1,135,974,172	126,640
GL 11	Spike	5.74	0.41	1.94	1.74	1,633,401,188	200,017
	Recovery	1.76	.41	1.27	1.74		
GL 13	Reference	2.29	2.02	BDL	0.71	285,458,166	120,161
GL 16	Spike	3.2	3.37	0.68	1.04	185,660,183	102,653
	Recovery	0.91	1.35	0.6	0.33		
ML 18	Reference	BDL	0	BDL	0	61,582,171	-
ML 19	Duplicate	BDL	0	BDL	0	63,775,191	-
Mean St. Dev.		0.08 0.01	0 0	0.08 0.0	0 0	62,678,681 1,550,700	-
Coeff. Var.		6.72	-	0.41	-	2	-

*no data due to vial breakage during freezing

Technical System Audits

A TSA is a thorough, systematic, on-site qualitative audit of sampling and measurement processes and procedures associated with a specific technology demonstration. The objectives of the TSAs conducted during this demonstration were to assess and document the conformance of on-site testing procedures with the requirements of the Test Protocols, the ACT Quality Assurance Project Plan (QAPP), and associated Standard Operating Procedures (SOPs).

The TSAs were conducted in accordance with the procedures described in EPA's *Guidance on Technical Audits and Related Assessments for Environmental Data Operations (EPA QA/G-7)* and ISO 19011, *Guidelines for Quality and/or Environmental Management Systems Auditing*. The ACT Manager follows a checklist, which merges elements of checklists used for EPA, ISO 17025, and TNI Field Sampling and Measurement Organization (FSMO) assessments, to verify compliance with test requirements. The full TSA procedure is described in the ACT SOP *Technical Systems Audit Standard Operating Procedures.*

The TSA assessed ACT personnel, the test and analytical facilities, equipment maintenance and calibration procedures, sample collection, analytical activities, record keeping, and QC procedures. Reference sample handling and chain-of-custody were observed during each audit. Audit criteria were based on the Test Protocols, dated May 14, 2018, the ACT QAPP, and the EPA, ISO, and TNI standards.

The TSAs included observations of the following general areas:

- Quality Assurance
  - Adequacy of procedures.
  - Adherence to procedures.
- Personnel
  - Appropriate qualifications and knowledge of the requirements of the test.
  - Chain of command regarding description of assignments and specific duties.
- Sample collection

  - Sample containers and equipment (pumps, tubing).
  - Sample handling, including subsampling.
  - Sample transport and storage.
- Sample Quality Control
  - Replicate samples.
  - Blank samples.
- Sample integrity
  - Sample identification and labeling.
  - Chain-of-Custody.
- Analytical procedures
- Document control and records
  - Logbooks.
  - Data sheets.

There were no negative findings from the TSAs for the field and laboratory tests, which were implemented consistent with the Test Protocols, QAPP, and SOPs. Minor deviations were documented in laboratory records. There were no deviations which may have had an effect on data quality for these tests. For all tests, the implementation of the audited tests was performed in a manner consistent with ACT data quality goals. All samples were collected as described in the Test Protocols and SOPs. Examination of maintenance and calibration logs provided evidence of recent and suitable calibration of sampling and analytical equipment. The overall quality assurance objectives of the test were met. ACT personnel are well-qualified to implement the evaluation and demonstrated expertise in pertinent procedures. Communication and coordination among all personnel was frequent and effective. Internal record keeping and document control was well organized. The ACT staff understands the need for QC, as shown in the conscientious development and implementation of a variety of QC procedures.

Data Quality Review

Quality Control

Quality control samples collected included periodic duplicate field samples and field blanks to determine the adequacy and control of field collection and processing procedures of analytical laboratory processing and analysis procedures. QC samples were treated identically to routine samples in terms of sample identification, custody, request for analytical services, and data processing.

- Results from field blanks showed no contamination indicate that field procedures were adequate for accomplishing data quality objectives.
- If the concentration observed in a replicate did not meet the criteria for precision and accuracy, the value was rejected and a back-up sample was processed and analyzed.
- Calibration data were reviewed at a cursory level and were determined to be acceptable. No data qualification was required based on the calibration review.
- Custody for all reference samples, was adequately maintained throughout the collection, processing, and delivery of samples to the analytical laboratories. Chain-of-custody documentation was complete. All analysis holding times were met as described in SOPs for the method or the Test Protocols.
- Overall, data quality for the reference water samples was acceptable.

Data Verification and Validation

Data review is conducted to ensure that only sound data that are of known and documented quality and meet technology demonstration quality objectives are used in making decisions about technology performance. Data review processes are based in part on two EPA guidance documents: *Guidance on Environmental Data Verification and Data Validation* (QA/G-8) [EPA, 2002] and *Guidance on Technical Audits and Related Assessments for Environmental Data Operations* (QA/G-7) [EPA, 2000].

The data were verified and validated to evaluate whether the data have been generated according to the Test Protocols and satisfied acceptance criteria. Data verification evaluates the completeness, correctness, and consistency of the data sets against the requirements specified in the Test Protocols, measurement quality objectives (MQOs), and any other analytical process requirements contained in SOPs. Data validation assesses and documents compliance with methods and procedures and determines the quality of the data based on the quality objectives defined in the Test Protocols and QAPP.

The ACT QA Manager reviewed the reference data sets from all field and laboratory tests. The number of reference samples collected at each site and the laboratory tests are in Table 6. A total of 81 reference samples were collected for the field and laboratory tests. These included field duplicate and blank samples and matrix spikes. Each reference sample was split into replicates for ELISA, LCMS-LR, and qPCR analysis and phytoplankton cell counts. Replicate samples were split according to the analytical method.

Table 6. Summary of reference samples and analytical measurements performed for the current Technology Demonstration.

Site	No. of Samples[1/]	No. of Replicates Analyzed per Sample[2/]	No. of Measurements									
			ELISA[3/]			LCMS-LR			qPCR[3/]			Cell Counts[4/]
			W	D	F	W	D	F	W	D	F	
BGSU Laboratory	22	2	88	88	na	88	88	na	88	88	na	132
MLML Laboratory	17	2	68	68	na	68	68	na	68	68	na	102
Long Island Sound	11	2	na	na	44	na	na	na	na	na	44	66
W Lake Erie	8	2	32	32	na	32	32	na	32	32	na	48
Sandusky Bay	8	2	32	32	na	32	32	na	32	32	na	48
Monterey Bay	9	2	36	36	na	36	36	na	36	36	na	54
Pinto Lake	6	2	24	24	na	24	24	na	24	24	na	36

W: Whole water; D: Dissolved fraction; F: Filtered (particulate or intracellular).

1) Total field samples includes field duplicates, field blanks, and matrix spikes.
2) For each replicate field sample, for the duplicate LCMS-LR samples, one sample was shipped for analysis and one held back in case a second analysis was required. For the triplicate ELISA samples, 2 were analyzed and one held as back-up.
3) Each reference extract for ELISA and qPCR was subsampled into 2 or 3 wells on a plate.
4) Triplicate cell counts per replicate subsample.

The data verification determined that the sampling and analysis protocols specified in the Test Protocols were followed, and that the ACT measurement and analytical systems performed in accordance with approved methods, based on:

- The raw data records were complete, understandable, well-labeled, and traceable;
- All data identified in the Test Protocols were collected;
- QC criteria were achieved; and
- Data calculations were accurate.

Data validation uses the outputs from data verification and included inspection of the verified field and laboratory data to determine the analytical quality of the data set. Validation of the data sets established:

- Required sampling methods were used;
- Sampling procedures and field measurements met performance criteria; and
- Required analytical methods were used.

The data validation also confirmed that the data were accumulated, transferred, summarized, and reported correctly. There is sufficient documentation of all procedures used in the data collection and analysis to validate that the data were collected in accordance with the demonstration's quality objectives.

Audit of Data Quality

The ACT QA Manager also conducted an Audit of Data Quality (ADQ) on verified data to document the capability of ACT's data management system to collect, analyze, interpret, and report data as specified in the Test Protocols, QAPP, and SOPs. An ADQ involves tracing data through their processing steps and duplicating intermediate calculations. A representative set of approximately 10% of the data was traced in detail from 1) raw data from field and laboratory logs, 2) data transcription, 3) data reduction and calculations, to 4) final reported data.

The ADQ determined that the data were accumulated, transferred, reduced, calculated, summarized, and reported correctly. There is sufficient documentation of all procedures used in the data collection and analysis to verify that the data have been collected in accordance with ACT quality objectives defined in the ACT QMS.

Data Quality Assessment

The Data Quality Assessment (DQA), sometimes referred to as a Data Usability Assessment is a scientific and statistical evaluation of validated data to determine if the data are of the right type, quality, and quantity to support conclusions on the performance of the technologies. The DQA process includes consideration of:

- *Soundness* - The extent to which the scientific and technical procedures, measures, and methods employed to generate the information are reasonable for, and consistent with, the intended application.
- *Applicability and Utility* - The extent to which the information is relevant for the intended use.
- *Clarity and Completeness* - The degree of clarity and completeness with which the data, assumptions, methods, and quality assurance, employed to generate the information are documented.
- *Uncertainty and Variability* - The extent to which the variability and uncertainty (quantitative and qualitative) in the information or in the procedures, measures, and methods are evaluated and characterized.

The DQA determined that the test's data quality objectives, described in Section 7.2 of the Test Protocols and Section 3.4 and Appendix B of the ACT QAPP (ACT, 2016), were achieved. This evidence supports conclusions that:

- The sample design and methods met requirements for collection of representative samples.
- Deviations from the Test Protocols were necessary, documented, approved, and did not affect data quality.
- The achievement of the completeness goals for number of samples collected, and the number of sample results acceptable for use provides sufficient quality data to support project decisions. Sufficient samples were taken to enable the reviewer to see an effect if it were present as well.
- No sample results were rejected.
- The overall quality of the data is acceptable and the results, as qualified, are considered usable.

This evidence supports conclusions that:

- The sampling design performed very well and is very robust with respect to changing conditions.
- Sufficient samples were taken to enable the reviewer to see an effect if it were present.
- Data on the performance of the sensors are unambiguous, and a decision maker can make an informed determination on the performance of the test instruments with a high level of certainty.

REFERENCES

Agilent, 5991-4725EN. LC/MS/MS Analysis of Cylindrospermopsin and Anatoxin-a in Drinking Water Using US EPA Method 545**.**
http://hpst.cz/sites/default/files/attachments/5991-4725en.pdf

Berry, M.A., Davis, T. W., Cory, R.M., Duhaime, M.B., Johengen, T.H., Kling, G.W., Marino, J.A., Den Uyl, P.A., Gossiaux, D.C., Dick, G.J., Denef, V.J., 2017. Cyanobacterial harmful algal blooms are a biological disturbance to western Lake Erie bacterial communities, Environmental Microbiology, DOI: 10.1111/1462-2920.13640.

Brierley, B., Laurence Carvalho, Sian Davies, and Jan Krokowski. 2007. Guidance on the Quantitative Analysis of Phytoplankton in Freshwater Samples. Phytoplankton Counting Guidance_v1_2007 12 05.doc.

Kudela, RM. 2011. Characterization and Deployment of Solid Phase Adsorption Toxin Tracking (SPATT) resin for monitoring of microcystins in fresh and salt water. Harmful Algae, doi: 10.1016/j.hal.2011.08.006.

Loftin, K.A., Michael T. Meyer, Fernando Rubio, Lisa Kamp, Edythe Humphries, and Ed Whereat. 2008. Comparison of Two Cell Lysis Procedures for Recovery of Microcystins in Water Samples from Silver Lake in Dover, Delaware, with Microcystin Producing Cyanobacterial Accumulations. U.S. Department of the Interior, USGS, Open-File Report 2008–1341.

Mekebri, A, GJ Biondina, DB Crane. 2009. Method validation for microcystins in water and tissue by enhanced liquid chromatography tandem mass spectrometry. Journal of Chromatography A, 1216: 3147-3155.

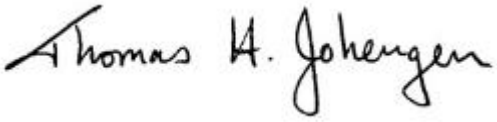
Miller MA, Kudela RM, Mekebri A, Crane D, Oates SC, Tinker MT, Staedler M, Miller WA, Toy-Choutka S, Dominik C, Hardin D, Langlois G, Murray M, Ward K, Jessup DA. 2010. Evidence for a novel marine harmful algal bloom: Cyanotoxin (microcystin) transfer from land to sea otters. *PLoS ONE* 5:e12576

ACKNOWLEDGEMENTS:

We wish to acknowledge the support of all those who helped plan and conduct the demonstration, analyze the data, and prepare this report. In particular, we would like to thank our Technical Advisory Committee, Dr. Joel Allen, U.S. Environmental Protection Agency, Dr. Dianne Greenfield, University of Southern California, Dr. Meredith Howard, Southern California Coastal Water Research Project, Dr. Keith Loftin, U.S. Geological Survey for their advice and direct participation in various aspects of this demonstration. Earle Buckley also provided critical input on all aspects of this work and served as the independent Quality Assurance Manager. This work has been coordinated with, and funded by, the National Oceanic and Atmospheric Administration, Integrated Ocean Observing System program.

September 27, 2020
Date

Approved By: Dr. Mario Tamburri
ACT Executive Director



September 27, 2020
Date

Approved By: Dr. Tom Johengen
ACT Chief Scientist

September 27, 2020
Date

Approved By: Dr. Earle Buckley
Quality Assurance Supervisor

September 22, 2020

MBio Diagnostics, Inc. Manufacturer's Response Letter

Alliance for Coastal Technologies Performance Verification Statement for MBio HAB Toxin MC/CYN Gen 1 System

MBio is grateful for the opportunity to participate in this ACT program, and we thank all the participants from ACT for this extensive evaluation of the MBio HAB Toxin MC/CYN System. This Gen 1 system was given to ACT for an evaluation concurrent with initial research use sales of the product. In addition to the commercially available MQ Reader and Gen 1 MC/CYN cartridges, MBio also supplied ACT with a prototype of a bead beating method of lysing cyanobacteria in the field and a battery to power the MQ Reader for field testing. MBio has subsequently been improving and productizing the lysis method and has developed Gen 2 MC/CYN cartridges with improved MC congener coverage. These MQ Algae Lyse and Gen 2 MC/CYN cartridges are planned for full commercialization in 2021.

MBio system results from the laboratory tests and field tests in Western Lake Erie were generally consistent with MBio's expectations, with reasonable correlation to LCMS and ELISA. As noted in the ACT Summary, differences in results from the laboratory reference methods ELISA and LCMS complicated the quantitative interpretation of the MBio results.

The California results were not as expected. The unusual false positive rate for the Pinto Lake samples was inconsistent with the Lake Erie arm of the study, and all other field tests performed by MBio. We therefore hypothesize that the cartridges used in California were somehow compromised during shipping or storage, as discussed below.

Comment on Reference Methods
ACT's reference methods for microcystin detection were LCMS and ELISA, consistent with US EPA Reference Methods 544 and 546. It is important to note that there were multiple examples where LCMS and ELISA did not agree quantitatively, although there was qualitative agreement in that samples with high levels of toxins measured high with both methods. Of note, samples from Sandusky Bay measured 4-8 times higher on ELISA MC than LCMS. Lack of quantitative agreement on the reference methods was surprising to MBio, as both methods are commonly used for determining microcystin levels in raw water samples. Verification of the MBio test was therefore in the context of the mismatched references. In general, MBio qualitatively agreed with both LCMS and ELISA, with generally closer agreement with ELISA.

MBio Microcystin Results for Laboratory Samples and Field Tests in Western Lake Erie
For cell culture samples and field samples from Western Lake Erie, the MBio results correlate with both ELISA and LCMS. Samples from Sandusky Bay measure significantly higher MC toxin concentrations on ELISA than LCMS. MBio's results correlate better with LCMS results for

5603 Arapahoe Avenue, Suite 1
Boulder, CO 80303
Phone: 303-952-2905
www.mbiodx.com

these samples, perhaps indicating that the ELISA results over-report toxin concentrations in this situation.

The precision trial indicates that the MBio System reports reliable and consistent results. Similarly, the analysis of a certified dissolved MC standard (BG18) was measured accurately on the MBio system.

From these data, MBio's prototype field-portable lysis system performed well on natural water samples containing cyanobacteria producing microcystin, since the measured dissolved and total toxin concentrations for Western Lake Erie are consistent with LCMS and ELISA results.

Congener equivalents are slightly different between the MBio Gen 1 test and LCMS as shown in the table below. MBio will soon launch a Gen 2 product with improved congener equivalents.

Congener Equivalents	RR	YR	LR	LA	LF	dmLR	LY	WR	NODR
LCMS	0.53	0.64	1	0.48	0.72	1	0.736	0.736	0.76
MBio Gen1	0.53	0.83	1	0.28	0.40	0.83		0.59	1

Cylindrospermopsin
In general, MBio does not recommend comparing toxin test results with reference tests that report nucleic acid copies rather than direct toxin measurements, as toxin production does not always correlate with gene copy number. Nevertheless, MBio CYN results compared well with the ancillary qPCR measurements, and since no reference toxin testing was performed, these results are the best available to assess the performance of the MBio CYN assay. As noted in the report, the measured CYN toxin concentrations on the MBio System correlate with the measured gene copies.

California, Pinto Lake
The results from California are unexpected, and we believe that the cartridges were damaged either in shipping between Ohio and California or in California. This damage is likely due to being exposed to high heat (above 37°C) for longer than a day. Since the ACT evaluation, MBio has added a time-temperature sensor to cartridge kit boxes to indicate whether the cartridges exceeded recommended temperature specifications.

Conclusion
MBio is grateful for being included in this ACT evaluation. This was an important milestone in our product development. Findings from this study directly informed product improvements. In the period since the ACT evaluation, MBio has moved forward with commercialization and has made a significant investment in advancing the platform for this important application.

5603 Arapahoe Avenue, Suite 1
Boulder, CO 80303
Phone: 303-952-2905
www.mbiodx.com