Ref. No. [UMCES] CBL 2020-021
ACT DS20-05

PERFORMANCE DEMONSTRATION STATEMENT
Beacon Field Tube Kit

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 Verification

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.

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EXECUTIVE SUMMARY

ACT conducted a performance demonstration of field-portable/-deployable assays, test kits, and sensor-based approaches that detect 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 applications in a diverse range of marine and freshwater coastal environments. We recognize 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.

The Beacon Field Tube Kits evaluated for this Demonstration are immunological tests for the quantification of dissolved and particulate-associated phycotoxins in marine or fresh water samples. Two tests were developed and submitted for evaluation: one commercial product to measure microcystins (Microcystin BX Tube Kit, #20-0098) and another beta version tube assay for domoic acid (DA), adapted from Beacon's Domoic Acid Plate Kit (#20-0249) designed for DA detection in shellfish homogenates. The Demonstration goals focused 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. Sampling ranges and analytical replication were often insufficient to establish clear statistical relationships between the Beacon Tube 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 Beacon kit 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 Beacon Tube Kit and reference methods differed among the natural test environments so there is likely congener-specific reactivities and matrix effects that should be evaluated more fully to better understand performance capabilities. For microcystin testing, differences in agreement among the two laboratory reference methods, ELISA and LCMS-LR, were also apparent across different field-testing sites.

The beta testing of the adapted Domoic Acid Tube Assay indicated that the HRP activity was strongly inhibited by high sodium exposure, requiring at least a 1:10 dilution of seawater media samples to obtain an accurate sample read. Lab testing of spike-in DA levels showed dissolved DA was consistently overestimated relative to predicted and LCMS measurements of DA. Particulate DA measurements were 0.01 to 0.002 X lower than dissolved DA indicating only a small carryover of media in the GFF samples. The efficacy of the Beacon DA Tube assay for determination of pDA loads was examined using a toxic *P. multiseries* in cultures of increasing cell abundance. Cell DA quota estimates derived from the Beacon DA Tube assay were not significantly different from the LCMS-DA derived estimates. Results also indicated that Beacon Whirlpak brayer homogenization was no less efficient than freeze thaw extraction for pDA.

In general the Beacon Tube Kit assays were easy to learn and simple to operate in both laboratory and field conditions. However, we note that the manufacturer's stated times for

tracer+sample incubations, sample washing steps, and color development were somewhat inconvenient for field assays, especially when multiple discrete sampling efforts are planned. We also note that the color development step must be done strongly shaded from sunlight as direct sunlight accelerates color development irrespective of toxin load, leading to underestimates.

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

There exists a need to expand the array of validated analytical techniques available for the quantification of dissolved and particulate-associated phycotoxins in marine or fresh water samples. Increased pressures for improved or expanded monitoring capability at minimal cost (given the increased frequency and negative impacts of harmful algal blooms) highlights the need for the development and implementation of reliable, field-based tests.

The Beacon Field Tube Kits are immunological tests for the quantification of dissolved and particulate-associated phycotoxins in marine or fresh water samples. Two tests were developed and submitted for validation: one commercial product to measure microcystins (Microcystin BX Tube Kit, #20-0098) and another beta version tube assay for domoic acid (DA), was adapted from Beacon's Domoic Acid Plate Kit (#20-0249) designed for DA detection in shellfish homogenates. The kits are designed to enable rapid, quantitative assessment of toxin loads in the field, subsequent to sample collection, i.e. shore-side, without need of electricity and minimal need for

specialized equipment. Because of their simplicity, sensitivity and low cost, Beacon's Field Tube Kits have distinct advantages over other assay designs that make them attractive for use by individuals or regulatory agencies where those advantages outweigh the cost or availability of other methods.

The tube assay format is suited for toxin detection in whole water samples, following freeze-thaw lysis (USEPA 546), or dissolved and particulate fractions generated by GFF filtration of know sample volumes. Dissolved microcystins can be measured in the filtered volume directly if present at sufficient concentration. A significant salt inhibition of horseradish peroxidase (HRP) tracer activity was observed during development of the DA Tube assay, requiring a minimum of a 1:10 dilution of seawater sample in DI for valid results (D. Waggoner, Beacon Analytical; pers comm). For this performance demonstration, the development of a field compatible lysis protocol was required to enable assay of GFF captured particulates. Beacon designed a lysing procedure that utilized WhirlPak Homogenizer Filter Bags containing a piece of nylon mesh (7oz, 330µm sieve, #B01385), and an ink brayer (>2in width). Following filtration, the GFF filter is placed on one side of the polypropylene bag filter, a known volume of MQ or sample filtrate is added and the GFF is completely disrupted by rolling brayer over the WhirlPak bag and contained GFF filter. The resulting 'grindate' is subsampled without dilution and run in the tube assay. The presence of filter particulate has no impact on assay performance. For this study, the total volume of sample filtrate was returned to the WhirlPak bag so concentrations within the extract represent that of the natural sample.

The fundamental principles of the two Beacon Field Tube Kits are the same. The tests are competitive ELISAs based on the ability of rabbit anti-toxin polyclonal antibodies to bind both free toxin (in a sample or calibrator solution) and a toxin-enzyme conjugate (the reporter molecule). Toxin competes with toxin-enzyme conjugate for binding to a limited number of anti-toxin antibodies present in an incubation mixture. The anti-toxin antibody-conjugate complex formed during the incubation is in-turn bound by anti-rabbit immunoglobulin G (IgG) immobilized on the inner surface of a test tube in which the incubation occurs. After the incubation, a series of washes removes any unbound or non-specific surface-associated material from the tube. During a second incubation period in an enzyme substrate solution, the retained enzyme conjugate catalyzes the generation of a blue-colored product from the (uncolored) substrate. The extent of color development is proportional to the amount of toxin-enzyme conjugate specifically bound to the surface of the tube, and due to competition between sample toxin and toxin-HRP conjugate, color is inversely proportional to the amount of free toxin in the sample (or calibrator) solution. A sample containing a low concentration of free toxin enables the anti-toxin antibodies to bind many toxin-enzyme conjugate molecules. The result is a dark blue solution. Conversely, a high concentration of toxin in a sample competitively decreases the number of toxin-enzyme conjugate molecules bound by the antibodies, resulting in a lighter blue solution. Color development is quantified using a hand-held pocket spectrometer. Each analysis of unknown sample(s) is accompanied by an analysis of known negative and positive controls. A calibration curve (created either on-site or previously and stored in a laptop) translates color absorbance into units of toxin concentration. Through prior optimization of assay and reagent design, toxin in an unknown sample can be reliably quantified over an established range of toxin concentrations (10 to 100-fold). The entire process of sample preparation and analysis can be accomplished in 1.5 hours, enabling a timely response to the dynamic conditions of harmful algal blooms. Please see www.beaconkits.com for specific assay protocols.

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 HAB 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 detection assay 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.

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

Two laboratory tests were conducted to evaluate range, accuracy, and precision of detecting both freshwater and marine HAB species and their associated toxins. Freshwater HAB testing was performed at Bowling Green State University (BGSU) within the laboratory of Dr. Timothy Davis and marine HAB testing was conducted at Moss Landing Marine Laboratory (MLML) within the laboratory of Dr. G. Jason Smith. Each laboratory test lasted approximately one week in duration and assessed analytical accuracy of the assays compared to reference sample analysis which included independent detection of toxin concentrations using USEPA adopted ELISA methods and LCMS measurements conducted by Dr. Raphe Kudela using state of California certified protocols based on USEPA standard methods. In addition, independent qPCR of targeted HAB primers and/or microscopic counts of targeted HAB species were conducted by ACT personnel during each lab test.

The freshwater lab testing at BGSU took place from July 11-15, 2018. 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) 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.

The saltwater lab testing at the Moss Landing Marine Lab, CA took place from September 11-14, 2018 and October 1-3, 2018. The testing utilized MLML cultures for domoic acid (*P. multiseries* and *P. pungens* [non-toxic culture]). Cultures were maintained in appropriate growth media (f/2 for *Pseudo-nitzschia*) at 15ºC under a 14:10 h light:dark photoperiod in an environmental chamber illuminated at 142 µmol (photons) m^{-2} s^{-1} with standard F40 cool white fluorescent tubes. 0.2 micron filtered Monterey Bay seawater was utilized for culture propagation and dilutions. Domoic Acid (DA) detection trials were conducted using several dilutions (1:10, 1:50, 1:100 and 1:200) of a stock *P. multiseries* culture. In addition a "cold" (low/no DA production) *P. pungens* culture was used as a matrix for spiking in reference domoic acid standards at target concentrations of 0, 2.5, 20 ppb dissolved DA (dDA).

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 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. On several field sampling events 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). Saltwater sampling sites were conducted in Monterey Bay, on the central California coasts. These test sites coincided with location of a long-term HAB monitoring station in southern Monterey Bay off the Monterey Commercial Wharf (MWII, sccoos.org/harmful-algal-bloom/).

Reference Sample Collection and Analytical Methods

Reference samples were collected during all field and laboratory tests for direct comparison between test instrument and independently analyzed laboratory results. All samples were processed to analyze toxin concentrations, toxin-producing genes (where available), and phytoplankton abundance. Toxin concentrations were determined using liquid chromatography/mass spectrometry (LCMS) and independent enzyme-linked immunosorbent assay (ELISA) kits. Reference sample ELISA measurements were conducted by ACT staff at the University of Michigan using USEPA 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. Field blanks were conducted utilizing toxin-free, Type 1 deionized water. Independent field duplicates were collected on several occasions to assess overall replicability of sample collection, processing, and analysis.

Liquid Chromatography/Mass Spectrometry

Samples for toxin analysis by LCMS 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 LC/MS 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 Beacon BX.

EQUIV	**0.86**	**0.53**	**1**	**0.41**	**0.34**	**1**	**0.30**	**0.29**	**0.58**
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 an 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 TESTS

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 dilution of the lysate from (BG01) to one-third the original concentration 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 making a one-third dilution of the lysate generated from extraction in BG01.

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 1). 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.

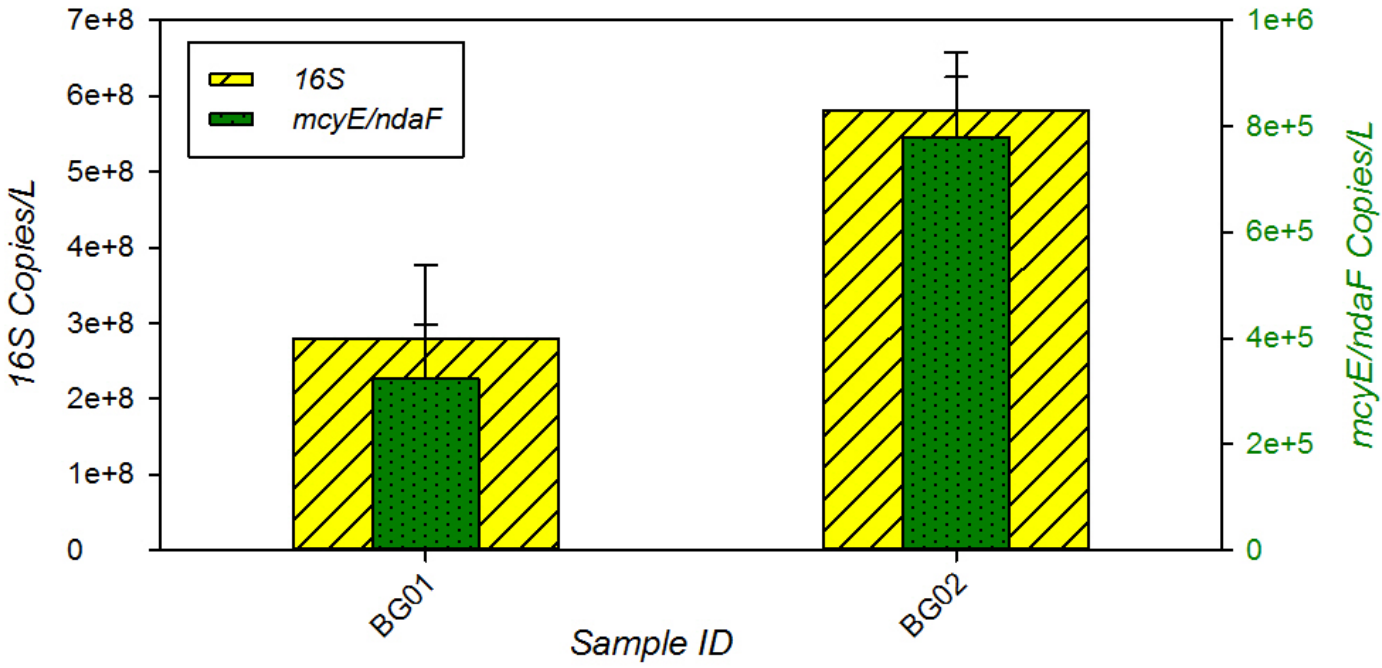

Figure 1. Estimates of cell density in gene copies per milliliter derived from the QuantaBio Q qPCR using the Phytoxigene CyanoDTec kit. Results are given for 16S total cyanobacteria copies per milliliter (left axis, yellow bars) and the mycE/ndaF (microcystin + nodularin) toxin gene copies per milliliter (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 Beacon for the common lysate test are shown against reference sample estimations based on laboratory ELISA and LCMS-LR (Figure 2). Only a single sample was produced for each concentration. Beacon results were in close agreement at the two lowest concentrations (BG01 and BG03) but under-predicted the concentration of BG02 (4.7 µg/L) compared to both ELISA (7.7 µg/L) and LCMS-LR (8.3 µg/L).

Although it was a very limited range and number of comparisons, cross plots of the comparative concentration estimates for the Beacon relative to ELISA and LCMS-LR concentrations are given (Figure 3; ELISA Cross stats: $R^2 = 1.0$, slope = 0.62; LCMS-LR Cross stats: $R^2 = 0.98$, slope = 0.54). The MC BX Tube Assay generally underestimated toxin abundance for this trial using a common lysate compared to the laboratory reference methods.

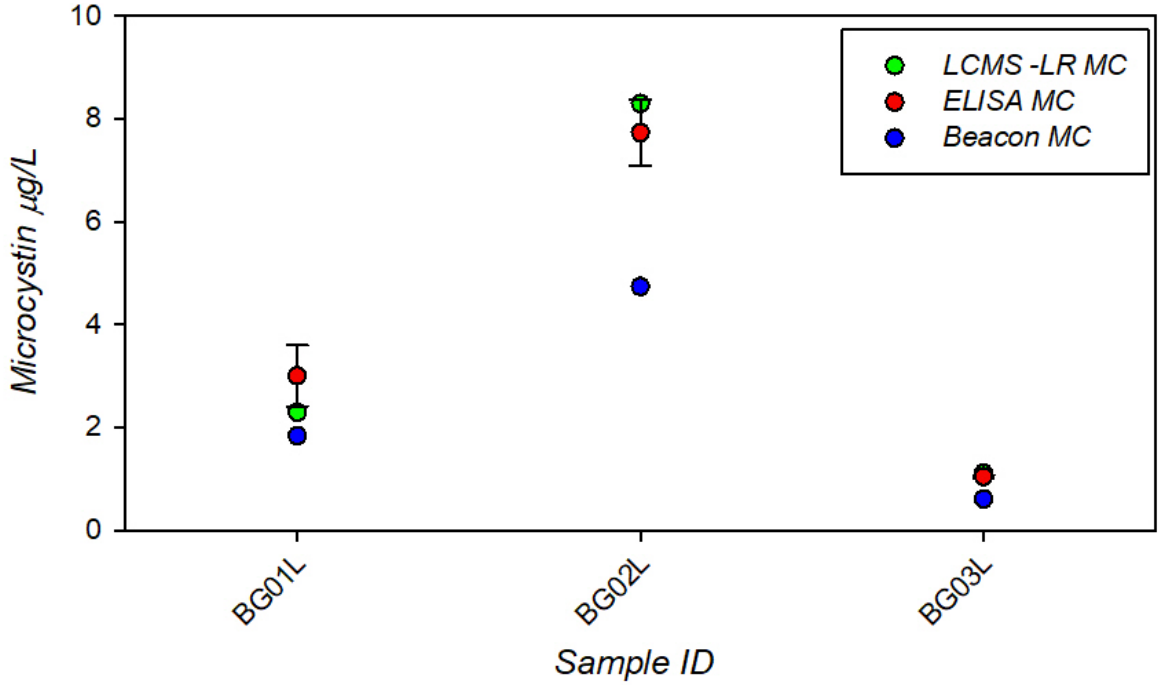

Figure 2. Comparison of microcystin estimates of samples analyzed by Beacon (blue), ELISA (red), and LCMS-LR (green) from the BGSU lab common lysate trial. Error bars are one standard deviation of the analytical replicates (n=2) for ELISA. LCMS-LR and Beacon have no error bars as there are no replicates.

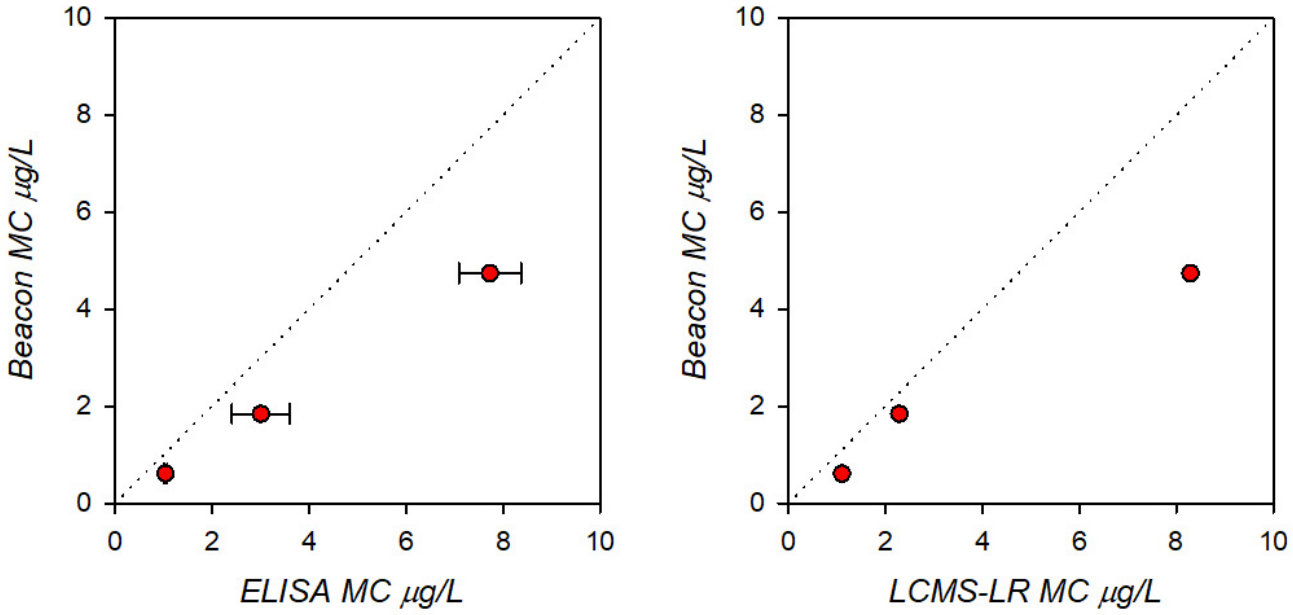

Figure 3. A cross plot of the Beacon measurements compared to reference ELISA (left) and LCMS-LR (right) for the common lysate laboratory trial. Error bars are one standard deviation of analytical replicates (n=2) for ELISA data, LCMS-LR and Beacon data has no error bars as there are no replicates.

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. The Beacon kit was not designed to measure the CYN toxin and only MC results are presented. 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 these generated reference samples are presented in figure 4. 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 Beacon results for microcystin concentration against the reference ELISA and LCMS-LR measurements is presented in figure 5. For five of the six test samples the reference LCMS-LR values were substantially higher than the ELISA values. The Beacon measurements tended 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.

Cross plots of Beacon and reference sample analysis estimates reveal a significant correlation of Beacon MC BX estimates with both reference methods (Figure 6. ELISA Stats: slope = 0.43, $R^2 = 0.92$; LCMS-LR Stats: slope = 0.29, $R^2 = 0.67$), although again the Beacon assay were consistently lower than the reference measures.

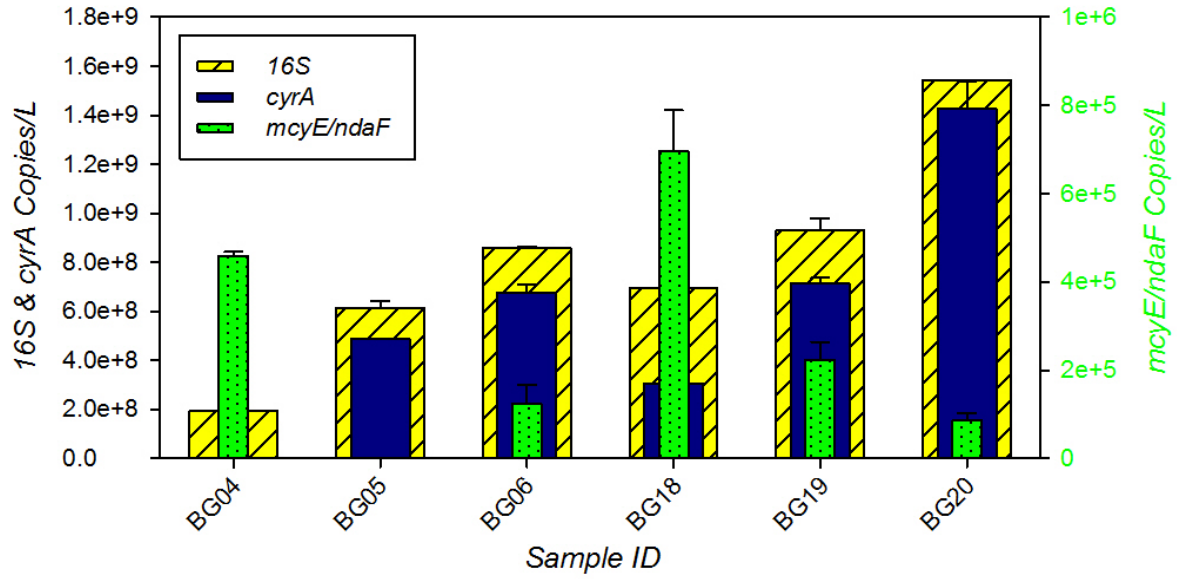

Figure 4. qPCR results for the mixed species lab test. Estimates of target gene markers in copies per liter derived from the QuantaBio Q qPCR using the Phytoxigene CyanoDTec kit. Results are given for 16S (yellow bars), mycE/ndaF (right axis, green bars), and cyrA toxin gene copies per milliliter (blue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

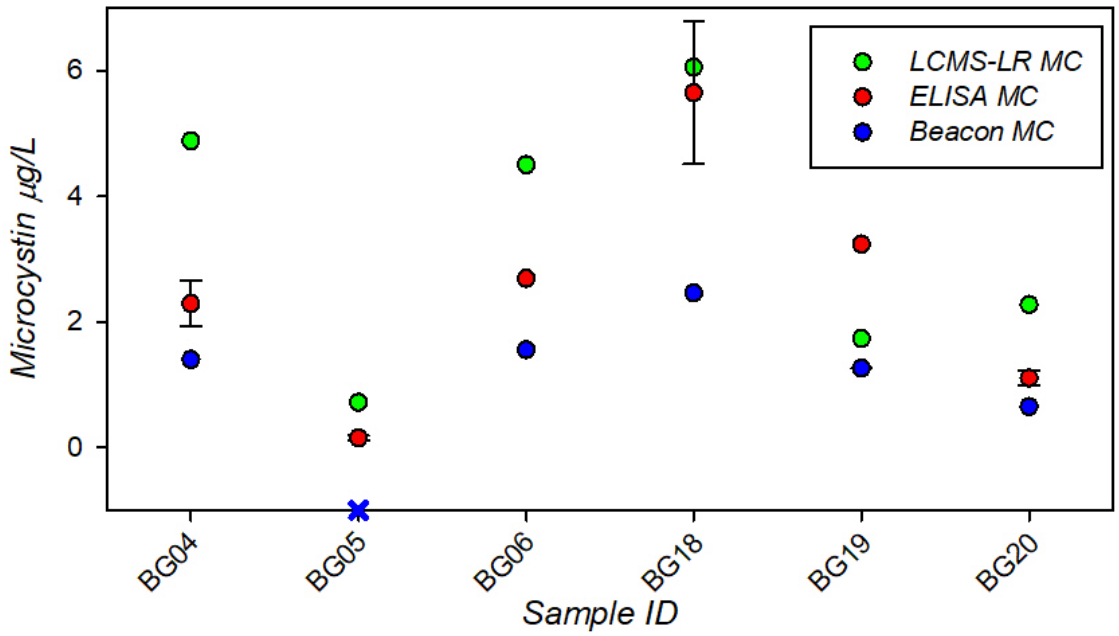

Figure 5. Comparison of MC toxin concentration measurements for the BGSU mixed species trial using *Microcystis* (LE3) and *Cylindrospermopsis* (CS-506). Cylindrospermopsin, associated with the cyrA gene was not measured in this study. Results are plotted for the Beacon test system (blue), reference ELISA (red) and LCMS-LR (green) microcystin data. Error bars are one standard deviation (n = 2) for ELISA, LCMS-LR and Beacon have no error bars as there are no replicates. BG05 resulted in a value below detection (BDL<0.3 µg/L) for the Beacon.

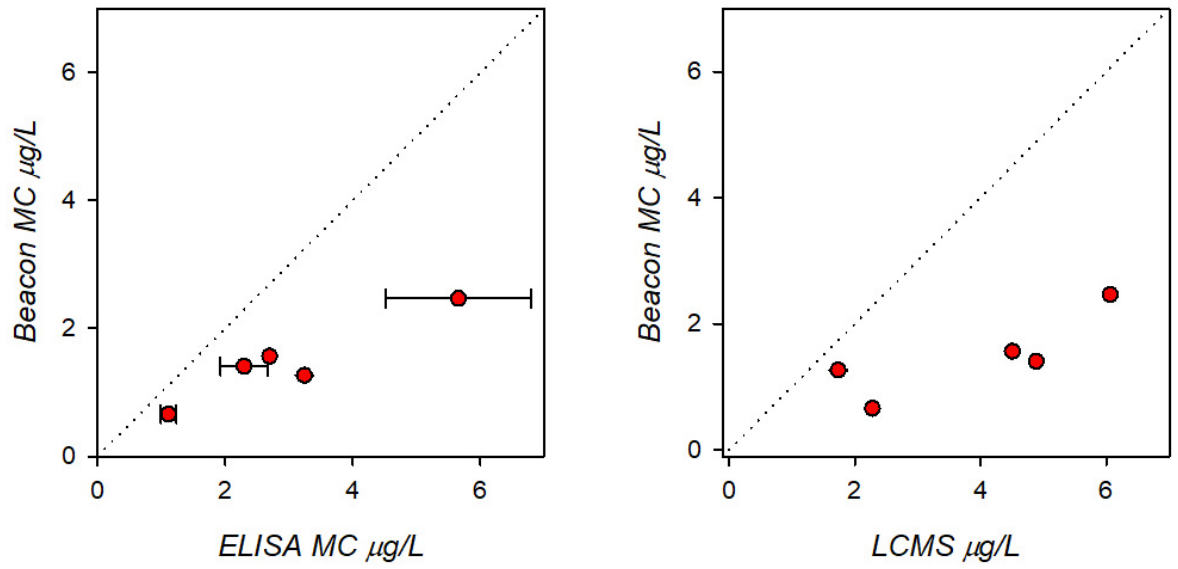

Figure 6. Cross plots for the Beacon measured microcystin compared to reference ELISA (left) and LCMS-LR (right) for the laboratory mixed species trial. Error bars are one standard deviation (n=2), LCMS-LR and Beacon data has no error bars as there was a single value. Beacon BDL data (<0.3 µg/L) is not included.

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 samples are shown in figure 7. 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 7) were relatively consistent across the mixtures (a factor of two), but somewhat inconsistent with the total

microscopic cell count data which declined across the test range (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.

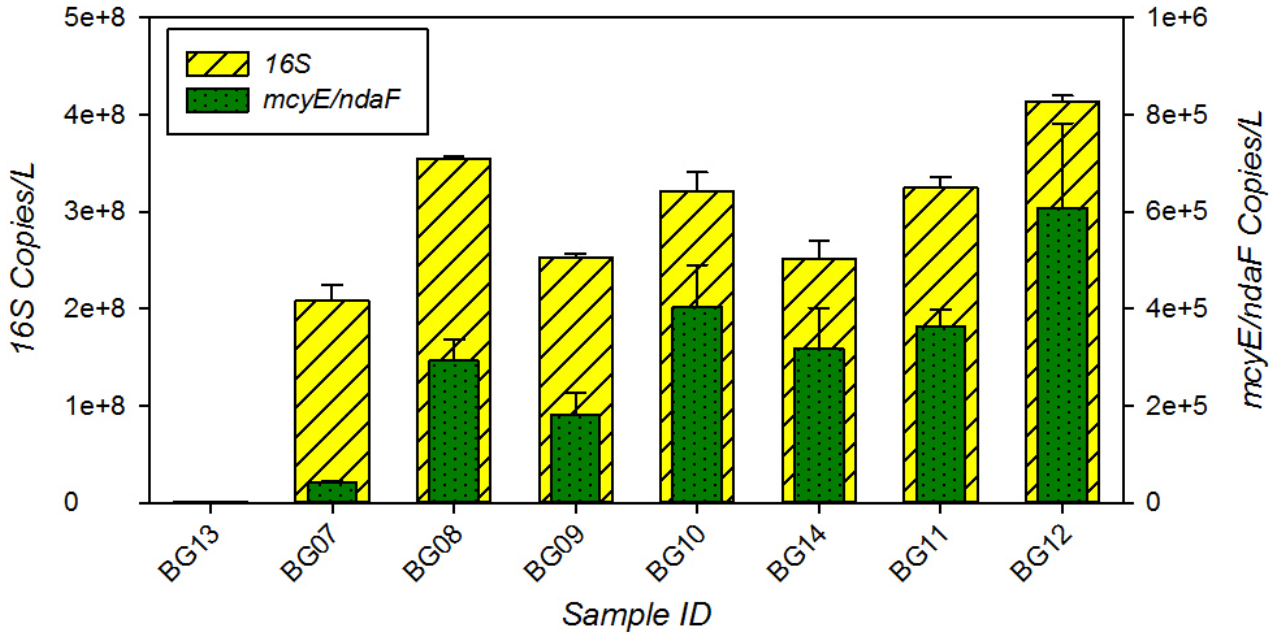

Figure 7. QuantaBio Q qPCR results for range trial. Samples BG10 and BG14 are duplicates in terms of the culture mixtures but were made independently. Sample BG13 was a media blank with no culture added. Error bars represent one standard deviation of the average for the two sample replicates and the two analytical replicates of a given sample extract (n = 4).

The Beacon MC estimations were significantly correlated with the ELISA and LCMS-LR reference measurements; however once again Beacon MC underestimated concentrations relative to the reference values (Figures 8 & 9). We note that toxin measurement at the highest concentration tested required a 3-fold dilution and recognize that matrix dilution may affect the various analyses differently. We also note that the reference method results for ELISA and LCMS-LR measurements themselves also diverged significantly at higher MC concentrations (Figure 8). Cross plots of Beacon and reference measurements are shown in Figure 9 and the linear regressions were significant for both ELISA comparisons (slope = 0.29, $R^2 = 0.93$) and for LCMS-LR comparisons (slope = 0.29, $R^2 = 0.67$).

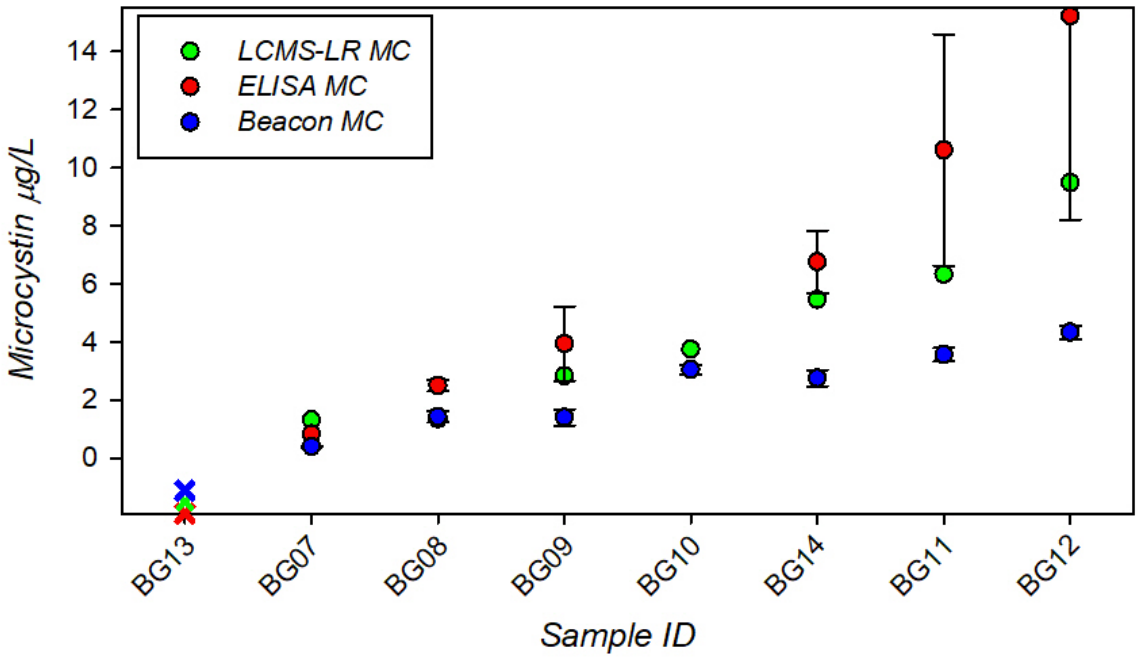

Figure 8. Comparison of microcystin estimates of samples analyzed by Beacon (blue), ELISA (red), and LCMS-LR (green) from the BGSU lab range trial. Error bars are one standard deviation (n=2) for Beacon and ELISA, LCMS-LR has no error bars as it is a single value. Values plotted on the X-axis represent values reported as below detection. Samples BG10 and BG14 are sample replicates made individually to check for consistency in both sample preparation and analysis, the ELISA sample for BG10 was lost due to breakage.

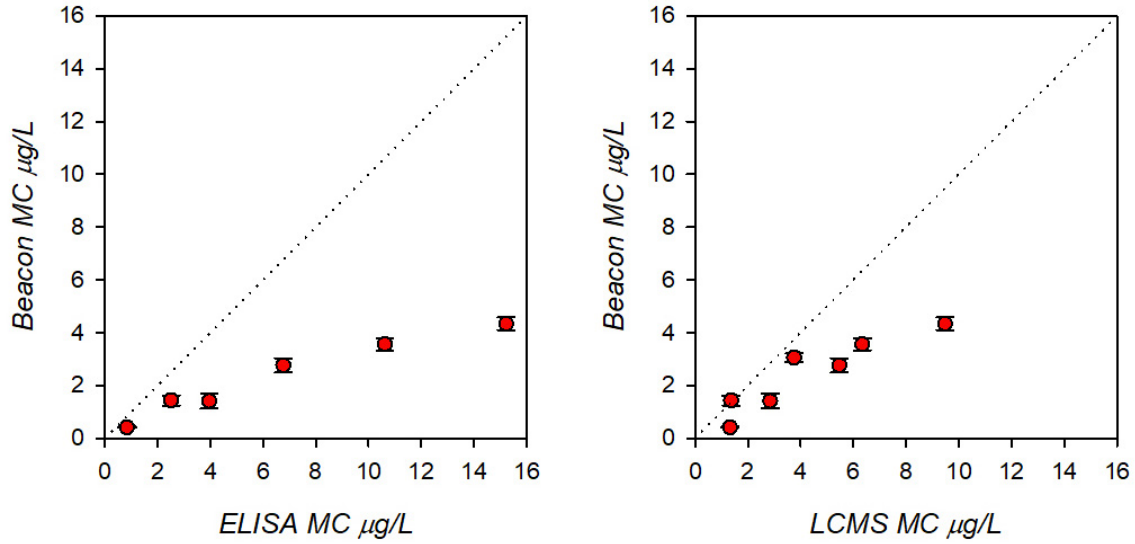

Figure 9. A cross plot of Beacon measurements compared to reference ELISA and LCMS-LR microcystin measurements for the laboratory range trial. Error bars represent one standard deviation (n = 2) for Beacon and ELISA, LCMS-LR have no error bars as there was a single value.

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 measurement 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 Beacon measurements ranged from 1.4 to 1.5 µg/L (mean = 1.4 µg/L, s.d. = 0.05). It is unclear why the LCMS-LR estimation was so much higher and there were no remaining back-up samples to re-analyze.

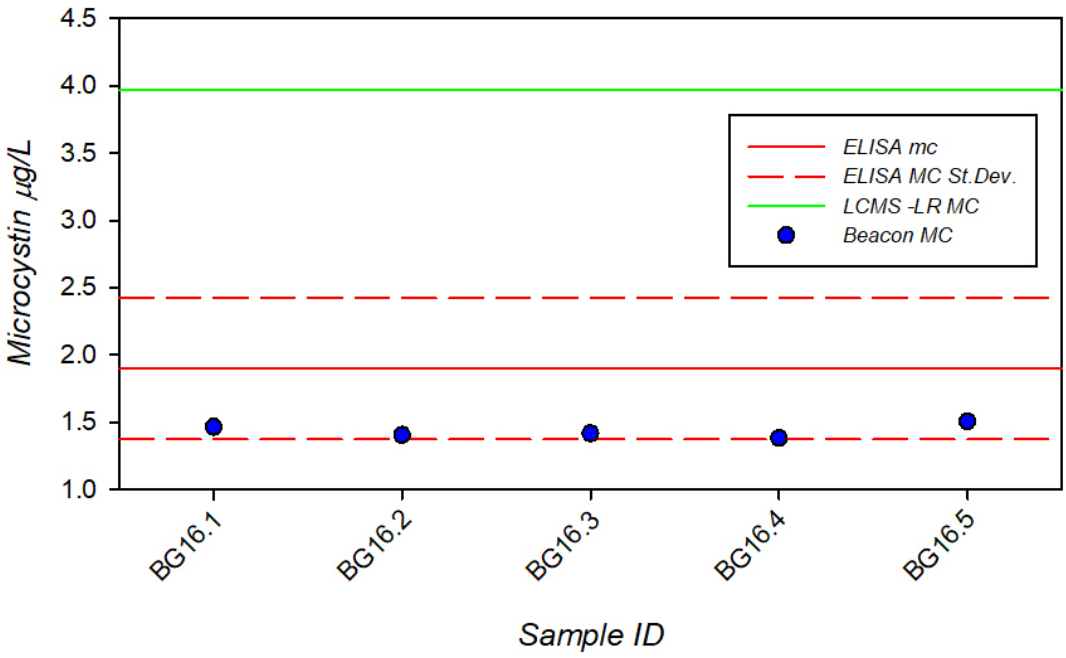

Figure 10. Results of the precision trial laboratory test using repeated measurement of a diluted certified MC standard to a targeted concentration of 1.5 µg/L, the ELISA estimated the concentration at 1.9 ±0.53.

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 Beacon measurement was 2.1 ±0.0 compared to an ELISA result of 1.29 µg/L and an LCMS-LR result of 3.41 µg/L.

RESULTS OF FIELD TESTS

Four freshwater field tests were conducted as part of the Beacon performance demonstration including Western Lake Erie and Sandusky Bay in the Great Lakes, and two inland lakes in central California, Pinto Lake near Watsonville and El Estero Lake near Monterey. 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 duplicate 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 11 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 11 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.

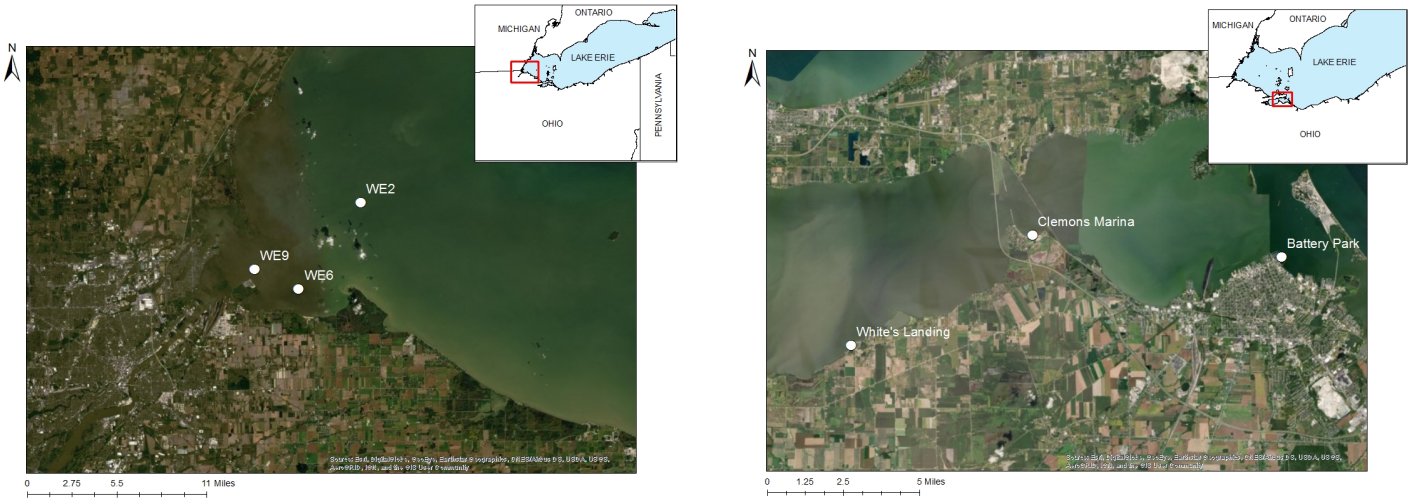

Figure 11. Western Lake Erie and Sandusky Bay sample stations. Western Lake Erie sampling stations (left) for WE2, WE6, and WE9. Sandusky Bay sampling locations (right) for Whites Landing, Clemons Marina and Battery Park.

Each Great Lakes field sample was processed and analyzed for both total and dissolved microcystin concentration. The total toxin measurements for Beacon were produced using the manufacturer's provided field lysing system followed by the tube kit reactions and immediate measurement on the field spectrometer. 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 12. 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 the NOAA Great Lakes Environmental Research Lab, the day prior to test sampling, because chlorophyll samples were not processed from that sampling event.

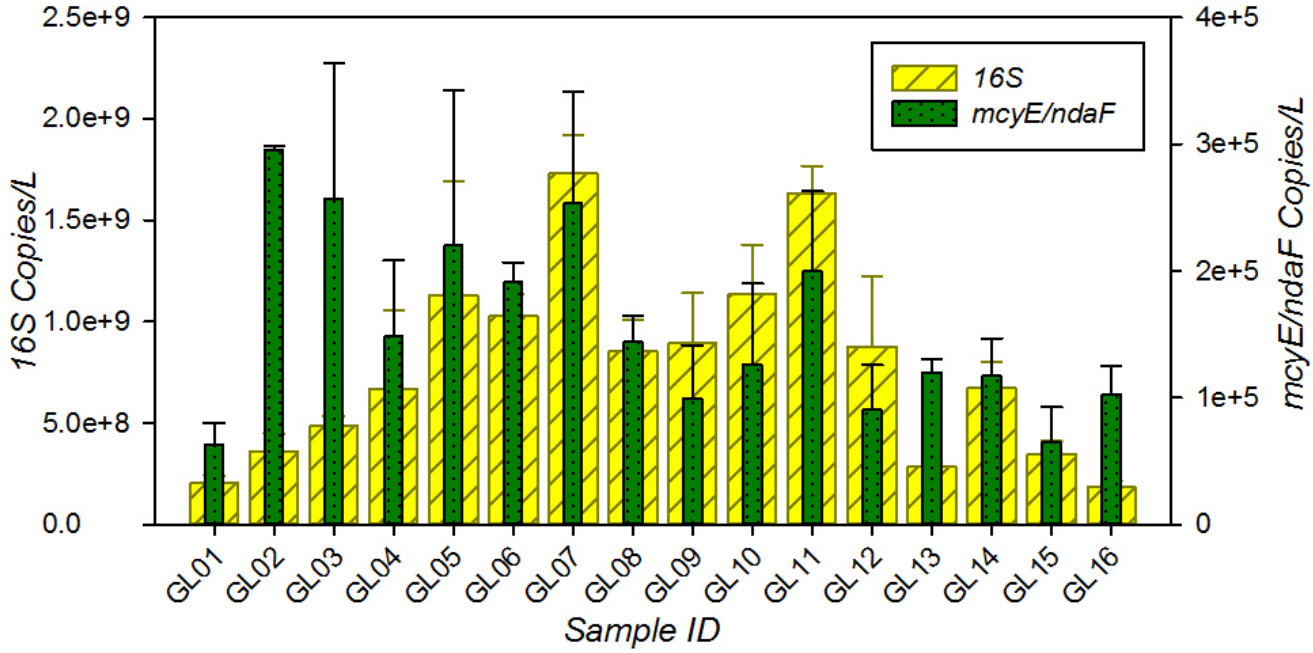

Figure 12. Great Lakes Field qPCR results for western Lake Erie (GL01-GL04 and GL13-GL16) and Sandusky Bay (GL05-GL12). GL02 & GL03 and GL06 & GL07 are independently collected field sample duplicates. Sample GL11 is a spiked addition of sample GL10 and sample GL16 is a spiked addition of GL13. Both spikes 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 Beacon and corresponding reference sample ELISA and LCMS-LR measured MC for the western Lake Erie and Sandusky Bay field trials is presented in figure 13. 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 comparisons of these samples are presented below in Table 5 of the QA/QC section.

There were distinct differences in the pattern of toxin estimates produced by the test and reference methods for the two different Great Lakes' environments (Figs 13-15). For western Lake Erie, dominated by *Microcystis*, methods were in closer agreement than observed in laboratory trials. For Sandusky Bay samples, enriched with *Planktothrix*, there was substantial divergence between assays with the reference ELISA MC measurements being 4 to 8 times higher than the LCMS-LR measurements and for the first time LCMS-LR estimates lower than the Beacon Tube estimates. It is likely that some of the MC toxin congeners produced by *Planktothrix* were not resolved by the adopted LCMS-LR analysis.

Overall the Beacon MC BX toxin measurements were correlated with, but lower than, both ELISA and LCMS-LR measurements of the western Lake Erie samples (Fig. 14; ELISA: slope = 0.41, R^2 = 0.57; LCMS-LR: slope = 0.36, R^2 = 0.74). For Sandusky Bay samples however, Beacon MC BX estimates were ca. 2x those of WLE tube toxin measurements but lower by 0.2 - 0.5x than reference ELISA measures and 2-10x higher than LCMS-LR estimates. Based on 16S qPCR

assays, total cyanobacterial abundance was 2-4x higher in Sandusky compared to WLE consistent with the higher measured Planktothrix load in Sandusky Bay and indicating that different water quality conditions may impact assay performance. Given the high degree of analytical variability and the variability in response across observed in the Sandusky Bay samples, correspondence of the Beacon MC BX tube assays with the reference assays were not significant (Figure 15; ELISA: slope = 0.11, R^2 = 0.20; LCMS-LR: slope = 0.01, R^2 = 0.00003).

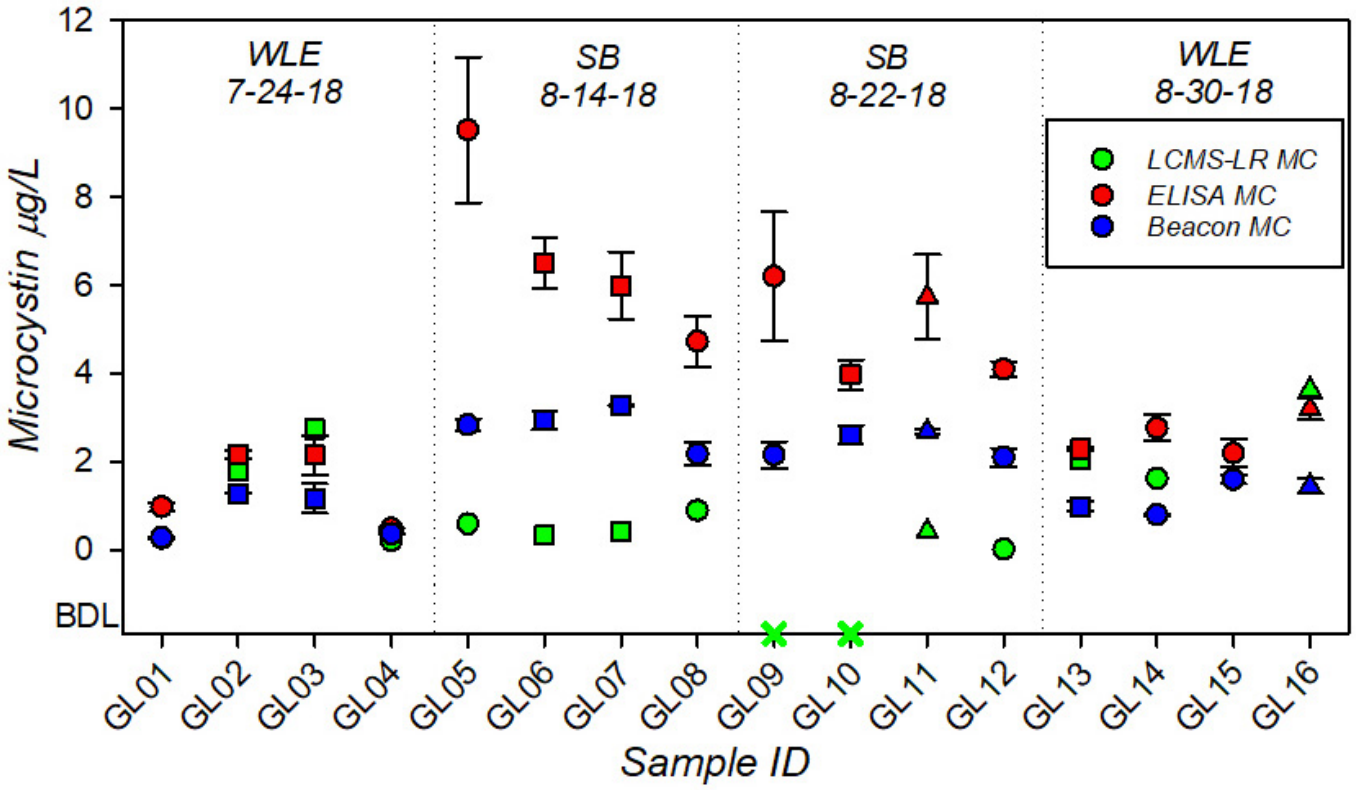

Figure 13. Great Lakes Field comparison of instrument (blue), reference ELISA (red) and LCMS-LR (green) total microcystin data. Samples GL 01-04 and GL 13- 16 were taken in western Lake Erie, samples GL 05-12 were taken in Sandusky Bay. Square data points represent duplicate samples, triangular, spiked samples, 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 Beacon and ELISA (n=2), LCMS-LR has no error bars as there was only one value.

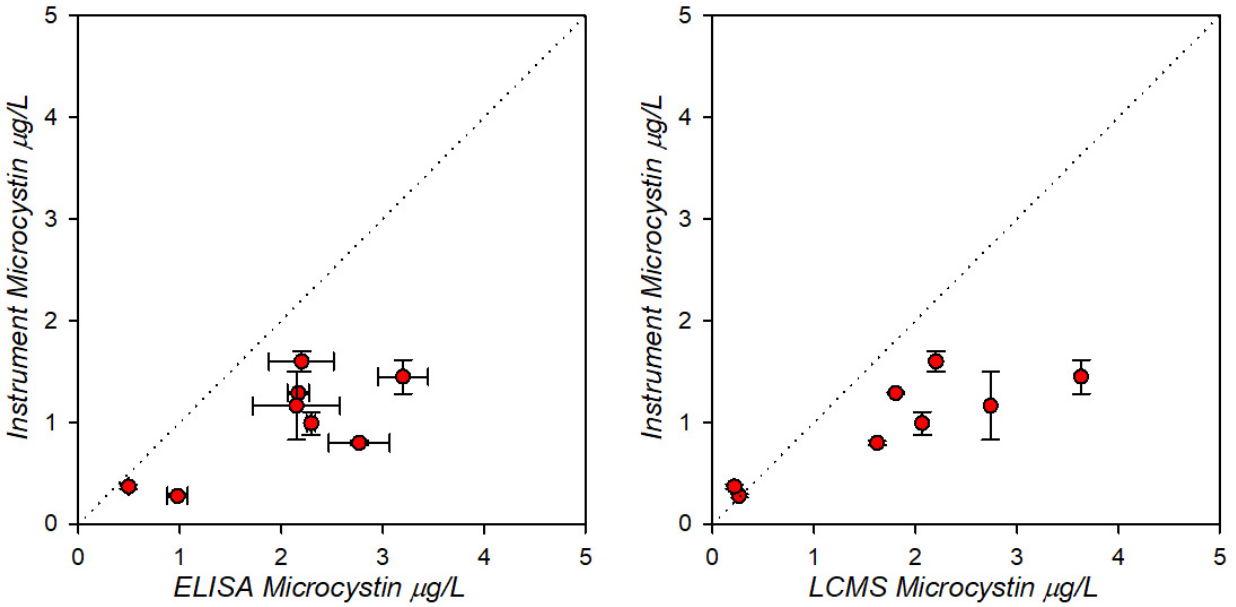

Figure 14. Western Lake Erie field response plot of Beacon compared to the ELISA (left) and LCMS-LR (right) whole water reference data. Error bars represent one standard deviation for Beacon and ELISA (n=2), LCMS-LR has no error bars as there was only one value. Data below detection is not included.

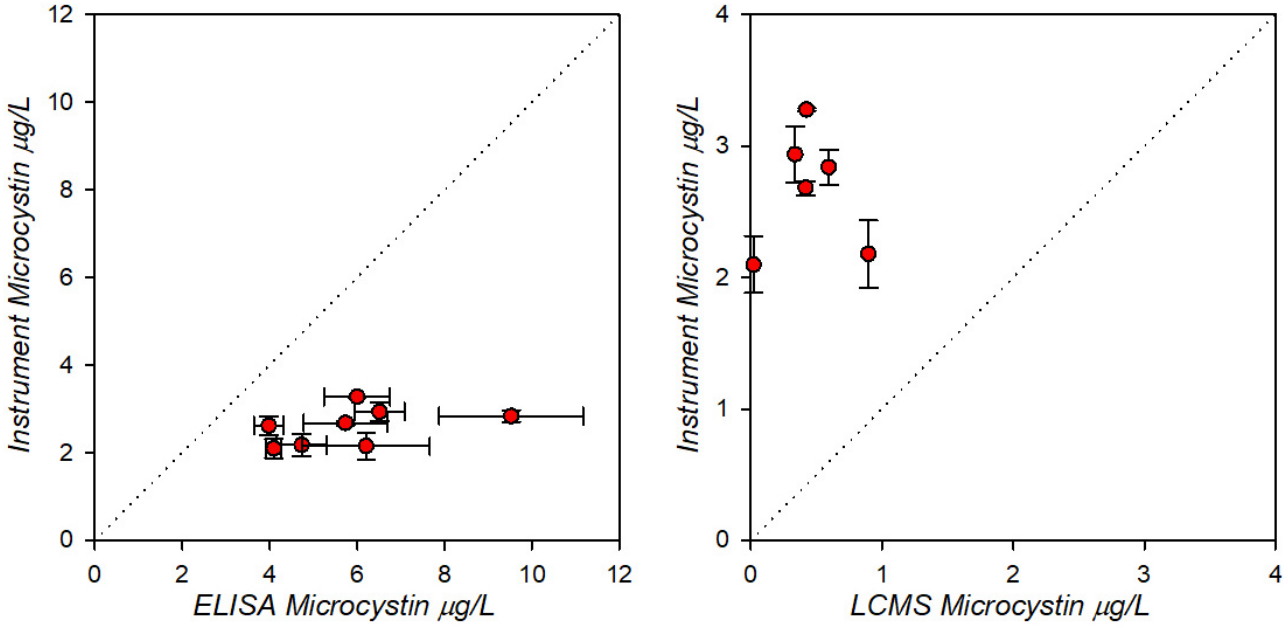

Figure 15. Sandusky Bay field response plot of Beacon compared to the ELISA (left) and LCMS-LR (right) whole water reference data. Error bars represent one standard deviation for Beacon and ELISA (n=2), LCMS-LR has no error bars as there was only one value. Data below detection is not included.

A time series of **dissolved** MC toxin measurements for the Beacon kit 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. In general, the dissolved MC represented only a fraction

(< 20 %) of the particulate microcystin fraction; and in 9 out of 16 cases was at or below the stated detection limit for the Beacon MC BX assay. This finding was typical for the active growth stage of cyanobacterial blooms with the bulk of toxin associated with particulate fraction. Beacon MC BX estimates where always lower than the reference measures. However, 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.

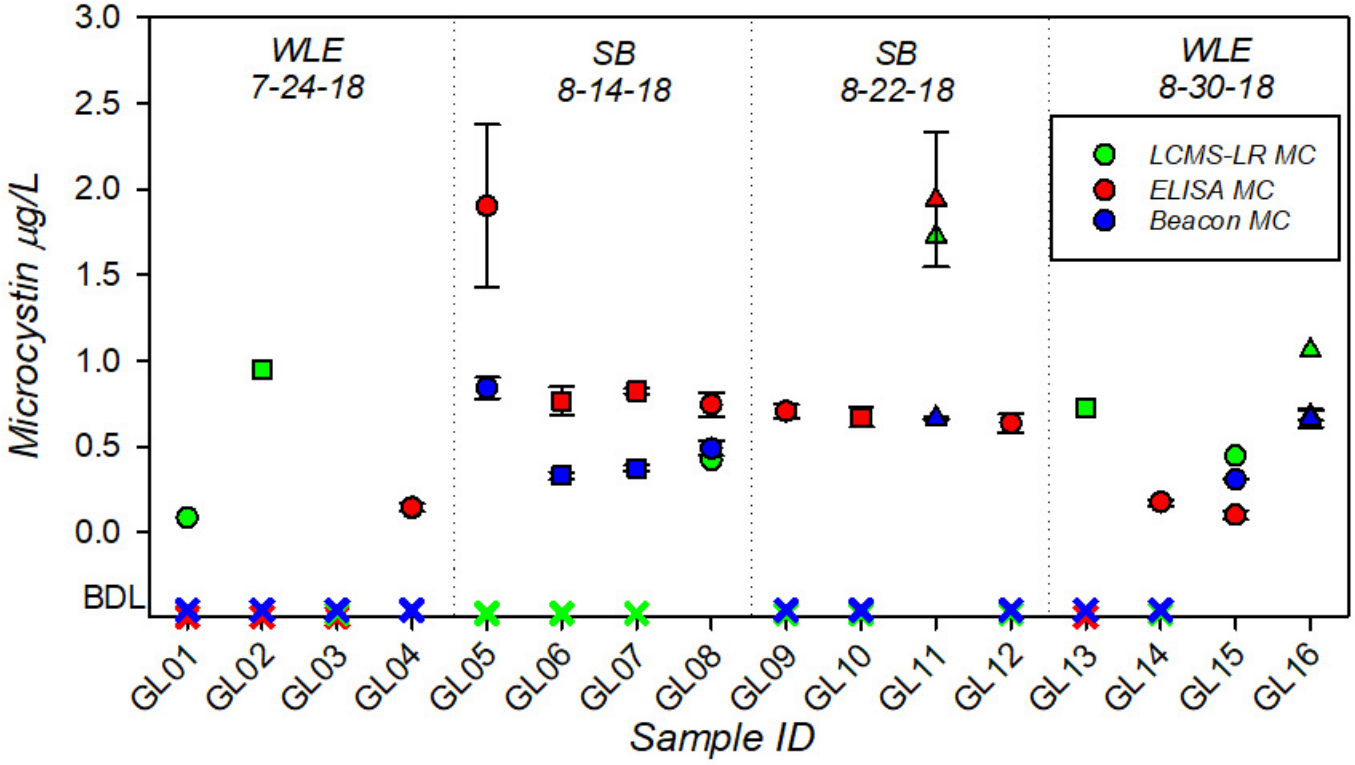

Figure 16. Great Lakes Field comparison of Beacon MC BX Tube (blue), reference ELISA (red) and LCMS-LR (green) assays of the dissolved microcystin fraction. Samples GL 01-04 and GL 13- 16 were taken in western Lake Erie, samples GL 05-12 were taken in Sandusky Bay. Square symbols represent independent duplicate samples, triangular symbols represent spiked duplicate samples, and crosses represent samples reported below detection. Error bars denote one standard deviation for Beacon (n=2) and ELISA (n=2), LCMS-LR has no error bars as it was a single value. Crosses represent data below detection, Beacon BDL < 0.3 µg/L, ELISA <0.1 µg/L, LCMS-LR BDL varies by congener all <0.5 µg/L.

California Inland Lakes: Pinto Lake and El Estero Lake

Microcystis aeruginosa, *Cylindrospermopsis* and *Planktothrix* were not observed at detectable levels at 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 generated 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.

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 17).

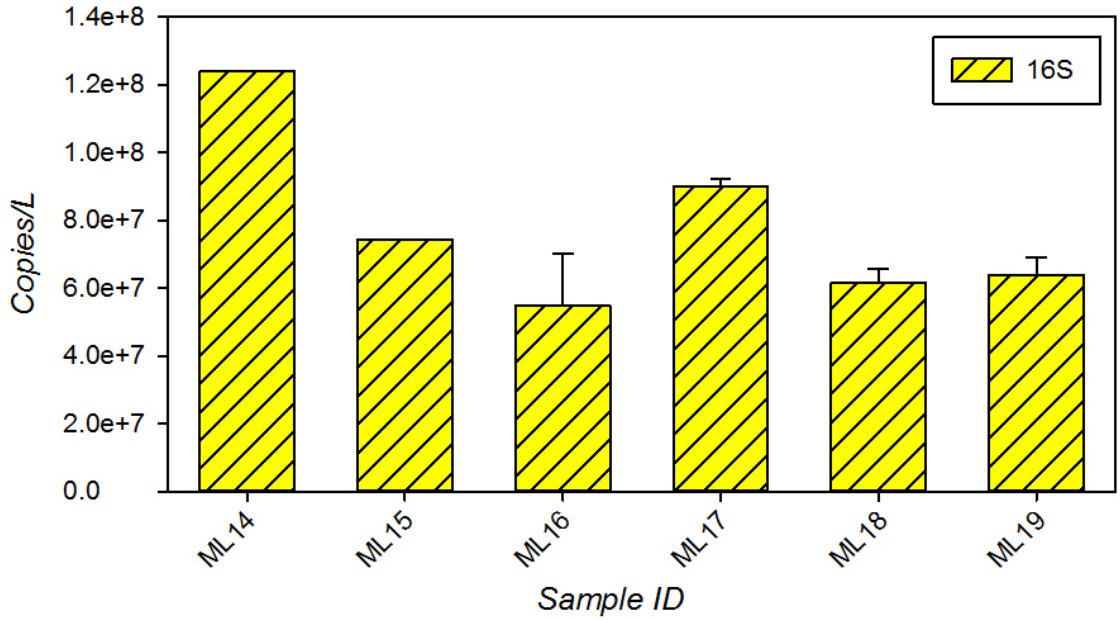

Figure 17. qPCR results for Pinto Lake and El Estero Lake (ML 15) reference samples. Copies of the mcyE/ndaF gene markers were all below quantification (< 45 copies per reaction) so no data are presented. Error bars are one standard deviation of two filters and two analytical replicates (n = 4).

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 Beacon estimated whole water MC concentrations less than 0.04 µg/L for samples ML14 through ML19 which is well below Beacon's defined BDL of 0.3 µg/L.

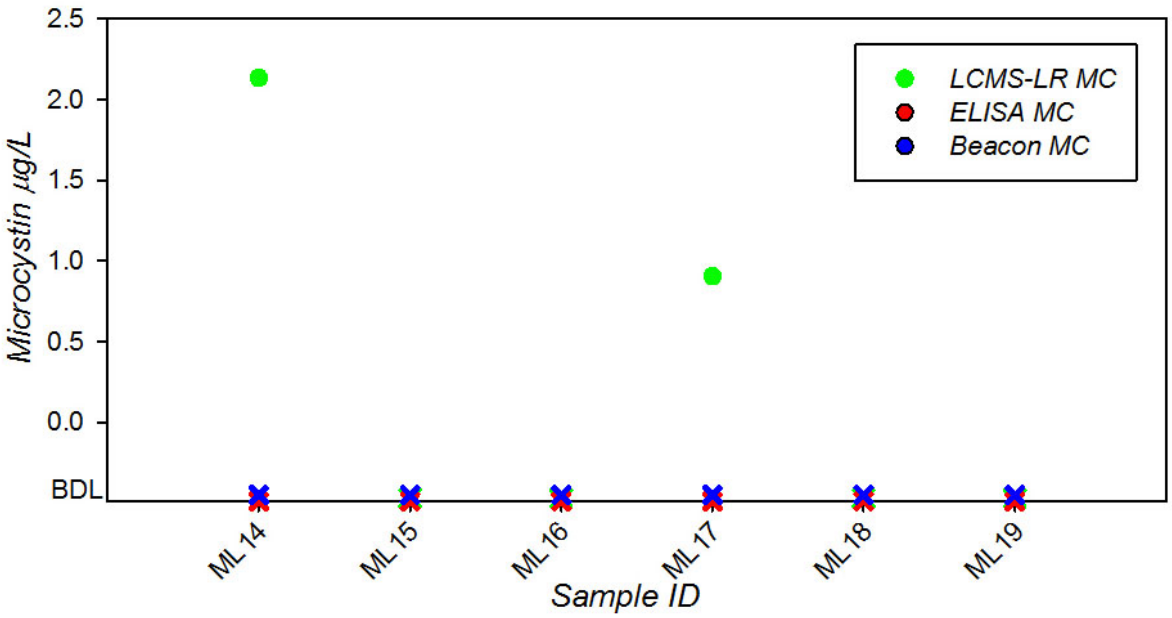

Figure 18. MLML Pinto and El Estero Lakes (ML 15) comparison of instrument (blue), reference ELISA (red) and LCMS-LR (green) whole microcystin data. Error bars denote one standard deviation for Beacon (n=2) and ELISA (n=2), LCMS-LR has no error bars as it was a single value. Beacon BDL < 0.5 µg/L. Samples ML18 and ML 19 are independently collected field duplicates.

DOMOIC ACID TUBE ASSAY BETA KIT DEMONSTRATION

MLML Laboratory Trials

The Domoic Acid Tube Assay was developed by Beacon staff specifically for this ACT demonstration through adaptation of Beacon's Domoic Acid Plate Kit (#20-0249) reagents into a tube format. During the developmental process it was noted that the HRP activity was strongly inhibited by high sodium exposure, requiring at least a 1:10 dilution of seawater media samples to obtain an accurate sample read (David Waggoner, Beacon Analytical, pers. Comm.).

The saltwater lab testing took place at the Moss Landing Marine Lab, CA from September 11 – 14, 2018 and October 1 – 3, 2018. The domoic acid testing utilized MLML cultures of Monterey Bay isolates of *Pseudo-nitzschia multiseries* (DA producer) and *P. pungens* (non-DA producer) and two UCSC cultures for saxitoxin (putatively *Alexandrium catenella* that exhibit different toxin profiles, USCS pers. comm.). Cultures were maintained in appropriate growth media (f/2 +Si for *Pseudo-nitzschia* and L1 for *Alexandrium*) enrichments of 0.2 µm filtered Monterey Bay seawater were utilized for culture propagation and dilutions. All cultures were maintained at 15°C under a 14:10 h light:dark photoperiod in an environmental chamber illuminated at 100 µmol (photons) m^{-2} s^{-1} with standard F40 cool white fluorescent tubes. During the saltwater lab testing, a total of 16 samples were generated. Filtration and collection of supporting reference samples (LCMS, ELISA, qPCR and counts) were all conducted immediately following culture manipulations (dilutions, mixing, spiking) following sampling protocols described earlier.

As DA monitoring often targets soluble (dDA) as well as particulate associated DA (pDA) a trial was conducted to assess the salt effect on the DA measurement baseline. As per protocol, 50 mL aliquots of lab grade DI or 0.1X Instant Ocean (IO, 3.5S) were pushed by syringe through GFF filters in swedgelock holders, followed by 25 mL air to remove excess fluid. As per standard protocol GFFs were ground in either 1 mL DI or 1 mL 0.1X IO. The results indicate that color development and hence HRP activity was inhibited (25.6%; ca. 1 ng/mL overestimate) in samples from the dilute salt exposure treatments (Table 5). This salt dependent HRP reporter inhibition will cause significant overestimation of DA in media samples, unless severely diluted, making dDA measurements in this beta format suspect at best.

Table 5. Salt inhibition of DA Tube assay. Blank absorbance readings (B_o) and hence HRP activity is reduced in samples with salt (3.5S) exposed filter grindates. The color development inhibition (0.434 AU) results in ca 1 ng/mL overestimation of DA content in blank samples. N=3

	Blank Absorbance	
Treatment	***mean***	***s.d***
GFF + DI	1.695	0.033
GFF + 0.1X IO	1.261	0.094
Difference	0.434	

This salt effect was evident in DA standard spike-in trials. Here, a non-toxic culture of *Pseudo-nitzschia pungens* was used as the biological matrix. Culture aliquots of *P. pungens* (5816 ± 605 cells mL^{-1}) were supplemented with DA reference standards (NRC CRM-DA-d) to predicted concentrations of 0, 2.5 and 20 ng/mL dissolved DA. Media samples were diluted 1:10 with DI and 100 mL collected onto GFF and the filters brayer ground in 1 mL DI. Both sample types processed in parallel according to the DA Tube Assay protocol. Results presented in figure 19 clearly demonstrate that while congruent with the spike-in levels, dDA was consistently overestimated relative to predicted and LCMS measurements of tDA. pDA measurements were 0.01 to 0.002 X lower than dDA indicating only a small carryover of media in the GFF samples.

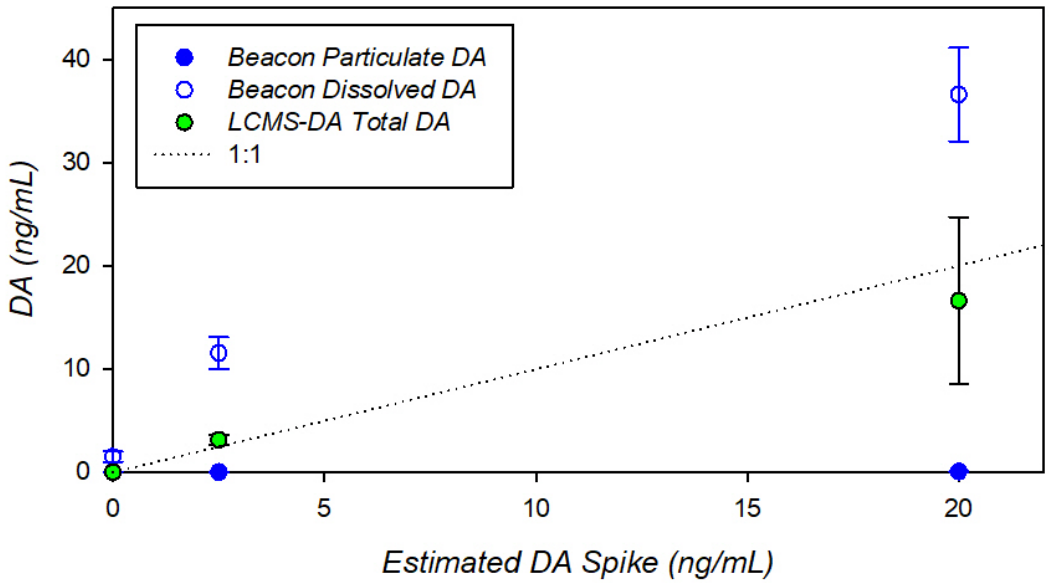

Figure 19. DA standard reference addition into non-toxic *P. pungens* culture. dDA is dissolved fraction, pDA is particulate fraction both measured by Beacon DA Tube Assay. tDA (i.e. dDA+pDA) was measure by LCMS-DA. Dotted line represents 1:1 recovery. Symbols are mean ± s.d., n =3.

The efficacy of the Beacon DA Tube assay for determination of pDA loads was examined using a toxic *P. multiseries* isolate in cultures of increasing cell abundance. Stock dilutions of 1:10, 1:50, 1:100 and 1:200 were used to assess linearity of tube assay response. Here 100 mL of culture was collected onto GFF filters and air purged filters brayer ground in 1 mL of DI. LCMS-DA was measured on replicate filters freeze thaw extracted in 1 mL DI. pDA loads scaled with increasing *P. multiseries* abundance or stock culture volume added (Figure 20 top) as did LCMS derived pDA reference measurements. Consequently cell DA quota estimates derived from the Beacon DA Tube assay were not significantly different from the LCMS-DA derived estimates (Figure 20 bottom). Results indicate that the Beacon Whirlpak brayer homogenization is no less efficient than freeze thaw extraction for pDA analysis.

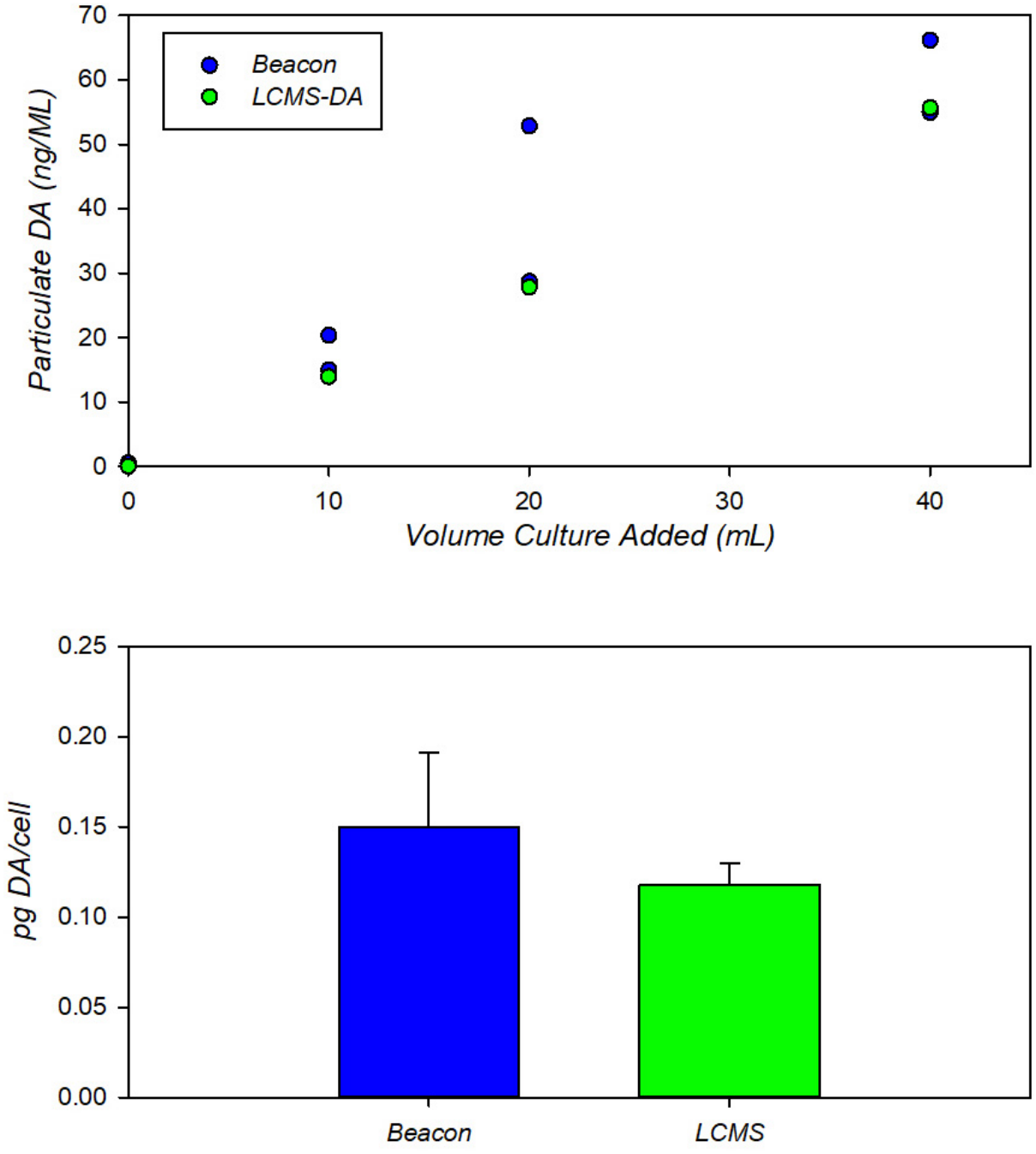

Figure 20. pDA analysis of toxic *Pseudo-nitzschia multiseries* laboratory cultures. *Top Panel:* Increasing volumes of a stock *P. multiseries* cultures were added to 0.2µ filtered seawater and 100 mL of well mixed culture were collected onto GFF filters. *Bottom Panel:* Normalization of pDA measurements to *P. multiseries* cell abundance in each sample yielded similar estimates of *P. multiseries* cellular DA quotas.

Field Trials at the Monterey Commercial Wharf

During the testing period, concentrations of target HAB species were low (as gleaned from routine weekly phytoplankton counts at both wharf locations) throughout Monterey Bay, therefore trials took place with water collected from the Monterey Wharf only (36° 36.22′ N, 121° 53.36′ W) on September 19th, 20th, 26th and October 4th, 2018. A 4-liter capacity Van Dorn sampler was used to collect water at a target depth and combined into one carboy for processing: 1-5 m integrated (3 samples), 1-2 m (1 sample), 1 m (1 sample), 2 m (3 samples), and 5 m (1 sample). A total of 9 samples were generated, including 1 field duplicate and 1 matrix spike with cultured *Alexandrium* cells (30 cells/ml final concentration).

Figure 21**.** Location of field trials on the Monterey Commercial Wharf (MWII). Samples processed either on wharf or adjacent parking lots.

Use of the Beacon DA Tube Assay in the field yielded pDA estimates from natural *Pseudo-nitzschia* communities following a similar correlation with laboratory assays of known toxic strains (Figure 22 top). Uncorrected dDA estimates were low and while yielding significantly lower HRP product absorbance than standard blanks (B/Bo = 0.819 ± 0.183, n = 18), 12 of 18 samples were below quantification level (BQL) based on lowest DA calibration standard (0.5 ng/L). Upon correction for dDA media dilution (10x), the dDA abundance swamped pDA concentrations in the field, ranging 10-20X higher (Figure 22 bottom). In contrast, LCMS tDA estimates were below detection for 3 of 5 reference samples; tDA was only observed at >700 *Pseudo-nitzschia* cells/mL and consistently lower than the Beacon dDA estimates for the field. These results reflect the salt effect, resulting in dDA overestimation at 1:10 dilutions of seawater by the Beacon DA tube assay. Accurate, but less sensitive dDA measurements with the Beacon assay will require greater than 1:10 dilutions of seawater samples with DI. In contrast, pDA estimates scale with natural *Pseudo-nitzschia spp.* abundance indicating a low abundance toxic populations were sampled during these trials.

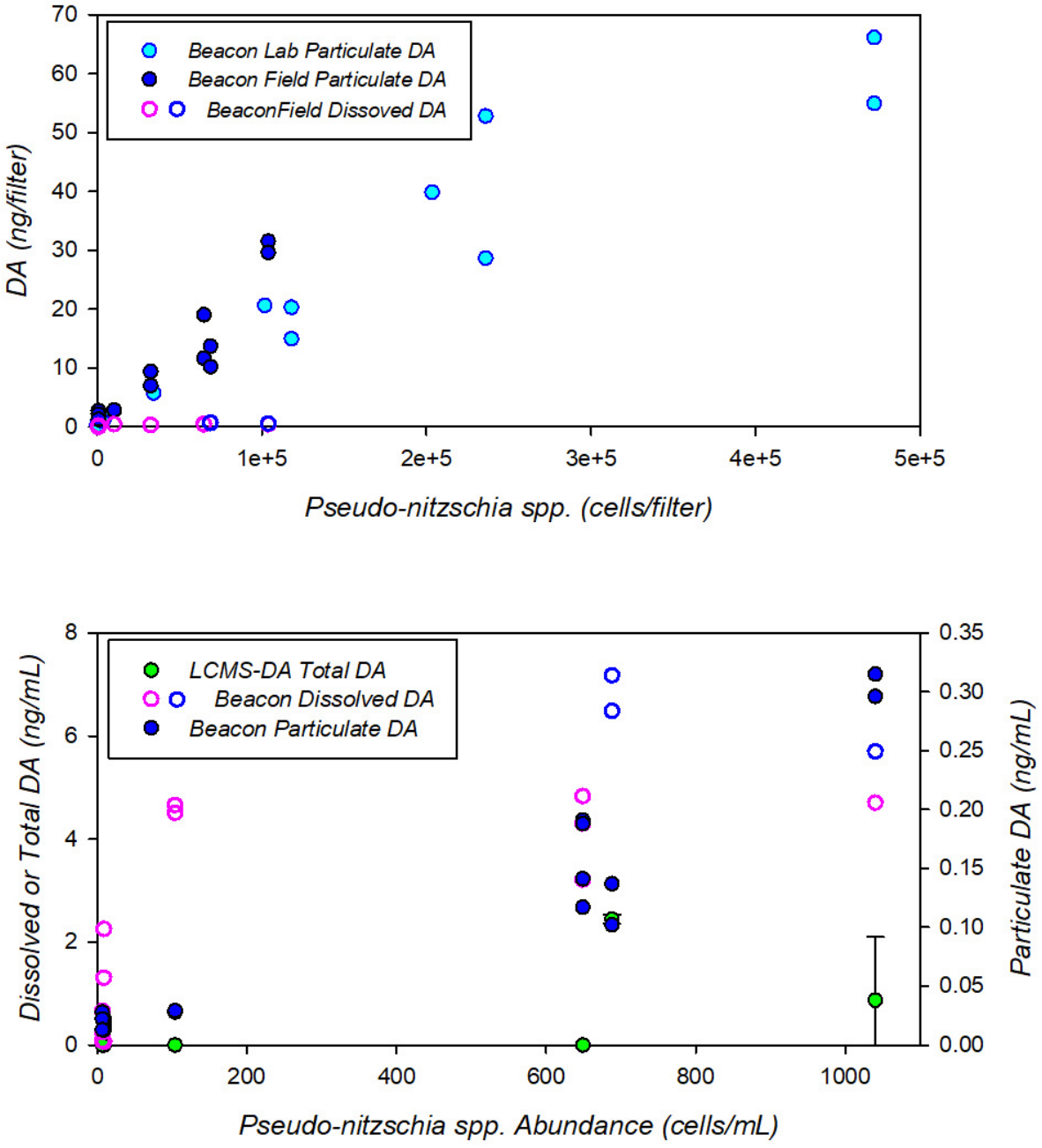

Figure 22. Performance of Beacon DA Tube Assay in field applications. *Top Panel:* Beacon pDA estimates scale with abundance of *Pseudo-nitzschia spp.* extracted. Filled blue symbols represent pDA measures on field samples, light blue symbols provide reference for pDA derived from known toxic *P. multiseries* cultures. Open symbols represent the dDA estimates for paired field samples (pink < BQL (0.5 ng/mL, blue = >BQL). *Bottom Panel:* Whole water DA fractions measured during Monterey Wharf II sampling. Solid blue symbols on the right axis represent whole water pDA levels (ng/mL), open symbols are dDA estimates plotted on left axis scale (pink < BQL, blue > BQL). Green symbols represent LCMS tDA measurements on paired water samples using right Y-axis scales.

Use Recommendations for Field Work

The protocol stated incubation times for tracer+sample incubations (30min) and color development (30 min) along with washing are somewhat inconvenient for field assays, especially when multiple discrete sampling locales are planned. Recommend decreasing these incubation times to 15 min when possible. In warm weather (>70^{o}F) color development proceeds rapidly and generally completes at <15 minutes. Color development must be done strongly shaded from sunlight as direct sunlight accelerates color development irrespective of DA load, leading to underestimates.

QUALITY ASSURANCE AND QUALITY CONTROL

All technology evaluations cond

ucted 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		6.76	5.04	1.82	1.65	286,496,352	360,548
St. Dev		-	1.18	0.03	0.02	49,383,586	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		2.16	2.01	0.08	0.47	422,595,885	276,482
St. Dev		0.01	0.84	0.02	0.66	86,719,143	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		6.25	0.38	0.80	BDL	1,378,076,368	222,184
St. Dev		0.36	0.06	0.04	BDL	497,347,940	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		0.08	0	0.08	0	62,678,681	-
St. Dev.		0.01	0	0.0	0	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 demonstraion. The objectives of the TSAs conducted during this demonstraion 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 demonstration 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.

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October 15, 2020
Date

Approved By: Dr. Mario Tamburri
ACT Executive Director

October 15, 2020
Date

Approved By: Dr. Tom Johengen
ACT Chief Scientist

October 15, 2020
Date

Approved By: Dr. Earle Buckley
Quality Assurance Supervisor

Company Response Letter, extracted from email to ACT Chief Scientist, Dr. Johengen on 10/13/20

Dear Dr. Johengen,

How are you? The following is my response to the report.

Page 5. The Beacon Domoic Acid (DA) plate kit (#20-0249) is designed to detect DA in shellfish homogenates. This kit was modified as a tube test for beta kit evaluation. The sample in this study was sea water which required higher kit sensitivity and sample dilutions. However the kit might exhibit salt interference. The original DA plate kit showed a recovery of 52.2 ug/g from a certified mussel tissue from NRC-Canada (49+/- 3 ug/g). The comparison of this DA assay with HPLC from Bigelow Analytical Services (East Boothbay, Maine) was around 115-132% (n=7), within the acceptable range.

The interference from salt indicates that the present assay needs modification (or adjustment). Again, the purpose of the DA plate assay was for Amnesic Shellfish Poisoning testing, if the salt water application for this field tube kit is required, Beacon will dedicate more time to improve the assay.

Page 6. It is uncustomary to have the test kits and reference standards coming from the same source in the study. Beacon is required to use the Certified Reference Material of Microcystin LR from an ISO 17034 certified organization, NRC (CRM-MCLR). The Domoic acid reference standard is from NRC.

Page 5. In order to use the stored calibration curve between different assay runs, a carefully designed format with controls built in to ensure integrity of the curve was required. The use of a stored standard curve is not recommended to users unless they are very skillful scientists with a good understanding of the assay, due the minor differences in techniques from one scientist to another and ambient temperatures from one day to another.

Page 11. The USEPA Method 546 and ADDA ELISA reference methods were used in this study. The results showed that the Beacon BX test underestimated the toxin concentrations. This is because the assay was developed for the detection of whole Microcystin LR molecules which is the most abundant (60-80%) and most toxic congener (Boyer, 2014). The assay showed less cross-reactivity towards other congeners. When field samples or whole water samples are not stored properly, microbial degradation of Microcystins will occur. The byproducts of Microcystin LR degradation will have no toxicity, as stated clearly in the paper published by

Harada (2004), however these molecules will be detected by the ADDA ELISA. This means that the ADDA ELISA will overestimate the toxicity and may cause public panic and increase of financial burden to treat drinking water. Nevertheless, if fresh samples are tested on site, this ADDA ELISA could be practical for the Microcystins monitoring purposes.

Page 32. The assay time could be shortened from 30 minutes to 15. It depends on the sensitivity requirement.

Page 32. Table 5, needs more explanation. Are these data from ADDA ELISA? GL06, 07, 10 and 11 ELISA vs. LCMS-LR are confusing.

These are some comments I have.

Thank you again for putting the data together.

Best regards,

Titan Fan, Ph.D.

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