



TECHNOLOGY DEMONSTRATION For Phytoxigene™ DinoDTec and CyanoDTec quantitative real-time PCR (qPCR) kits

TECHNOLOGY TYPE:	Field-Compatible Detection Kits for algal toxin genes
APPLICATION:	Field estimates of algal toxin genes for coastal environments
PARAMETERS EVALUATED:	Accuracy, precision, range response and reliability
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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 or species via immunological (i.e. antibody) and/or molecular methods. The fundamental objectives of this Technology Demonstration were to: (1) highlight the potential capabilities of commercially available quantitative polymerase chain reaction (qPCR) kits to quantify toxin genes of interest in cyanobacteria and saxitoxin-producing dinoflagellates; (2) verify the technology characteristics of these kits when tested in a controlled laboratory setting, and (3) verify technology characteristics of these kits 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 to definitively resolve differences among the manufacturer's test kit and the comparative laboratory reference analysis.

In this report we summarize the evaluation of two Phytoxigene™ real-time qPCR kits for the detection and quantification of algal toxin genes (versus active toxin production): 1) the CyanoDTec kit detects several toxin genes associated with cyanobacteria (microcystin/nodularin, cylindrospermopsin and saxitoxin) as well as ribosomal 16S; and 2) the DinoDTec kit detects the gene (sxtA) associated with saxitoxin production in dinoflagellates. Both kits are available commercially. The Demonstration goals focused on the ease of use in field applications and relative consistency of gene target determinations compared to standardized reference methods across different natural environments (i.e. quick environmental screening versus precision quantification for regulatory decisions). Controlled laboratory tests were included as part of the demonstration to help assess measurement ranges, and response to variable mixtures of toxic and non-toxic populations. qPCR copy numbers were consistently greater (typically an order of magnitude) from samples processed immediately using a quick bead beating and lysate dilution method versus matched reference samples that underwent a full Qiagen DNA extraction protocol. These multi-step, column-based protocols produce high quality DNA, but are known to result in loss of genetic material. This observation was consistent across the two kits and three different PCR platforms which further supports the conclusion that this loss in signal was related to the full extraction protocol. Unfortunately, field sites in California (freshwater and marine) were nearly devoid of toxic species during the testing period for this Demonstration. However, additional sites (Long Island NY, Great Lakes MI) provided environments where target toxin species were abundantly present.

Both Phytoxigene kits were easily adapted to all three qPCR platforms and in various laboratory and field settings. Furthermore, their simplicity of use was apparent in expert and non-expert hands alike. In use by ACT staff, the bead beating method and subsequent dilution of lysate were straightforward and preserved more of the genetic material compared to a full laboratory-based DNA extraction.

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 technology testing 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 coastal environments in order to provide users of this technology with an independent and credible assessment of instrument performance. To this end, the data and information on performance characteristics are focused on the types of information users most need. 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, 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/kits when tested in a controlled laboratory setting, and (3) verify performance characteristics of these instruments/kits when applied in real world applications in a diverse range of marine and freshwater coastal environments.

TECHNOLOGY TESTED

Phytoxigene's CyanoDTec kit simultaneously measures gene copies of total 16S ribosomal DNA, microcystin/nodularin, cylindrospermopsin (all three are hepatotoxins) and saxitoxin (a neurotoxin) from cyanobacteria in aquatic environment samples. The molecular kit is based on quantitative real time PCR (qPCR; Figure 1). Note, not all cyanobacteria species produce toxins, nor have the genetic potential for toxin synthesis, therefore the presence of an algal bloom does not immediately infer the risk of toxins being present. The CyanoDTec assay is made up of two separate tests (one for total 16S and an internal assay control, and a second for the toxin panel), each with its own set of reagents, packaged in separate aluminum foil pouches. The individual tests can be purchased separately depending on the application and algorithm chosen by the laboratory, but it is advised that at a minimum the Total Cyanobacteria assay (16S) be run on each sample to insure that the operator identifies any possible inhibition with the assay by utilizing the Internal Amplification Control (IAC) contained within this test. A set of nucleic acid standards (100, 1,000, 10,000, 100,000 and 1,000,000 specific gene copies per reaction) is available from Phyttoxigene for creating a standard curve for calculating specific gene copy numbers in unknown samples. The limit of detection for the assay, with a 95% degree of confidence is 45 copies in a reaction. Samples that are positive but below this level may be reported as positive by the instrument, however they have no value assigned to them. These results should be treated as 'Below

Quantifiable Limits' (BQL). The starting volume of the primary sample will have considerable impact on the overall limit of detection of the assay when reporting copy number per liter.

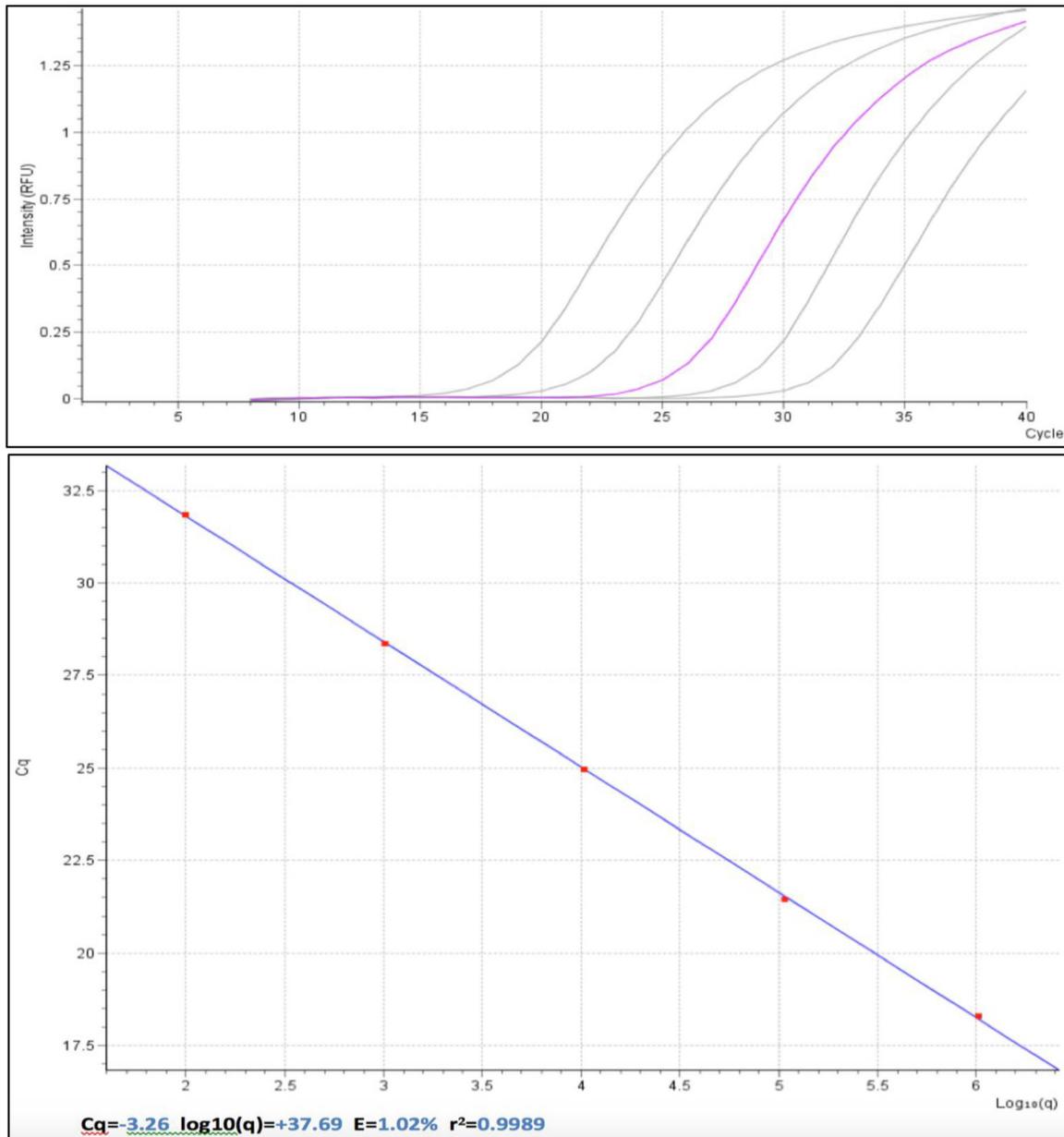


Figure 1. Progression of fluorescence from a 10-fold serial dilution of the microcystin/nodularin (mcyE/nda/F) standard (top panel), and subsequent standard curve used to quantitate unknown samples (bottom panel). PCR amplification is visualized by an increase in fluorescence resulting from liberation of a target-specific probe after each cycle (higher concentration of template = earlier fluorescence).

The extraction protocol involves concentrating samples onto filters and bead beating for 10 minutes using commercially available bead lysis tubes (BioGX, Birmingham, Alabama, USA). The resulting lysate is transferred to a sterile Eppendorf tube and stored on wet ice until qPCR set up. The lyophilized qPCR mix is reconstituted in 80µL sterile molecular biology grade water, and for

reaction set-up 20 μ L of that mix is combined with 5 μ L of the template. Any qPCR machine may be utilized with the kit, provided it contains three separate channels for excitation/detection of the following three fluorophores: FAM (495/516nm); Cy3/CalFluor Orange (538/559nm); and TxR/CalFluor Red (590/610nm). A MyGo Pro[®] (IT-IS Life Science Ltd.) was used for all CyanoDTec lab and field tests for this demonstration (Figure 2, left). The qPCR for all corresponding reference samples was run on a QuantaBio Q qPCR machine (Figure 2, right) as described below in the Reference Sample Collection and Analytical Methods section.

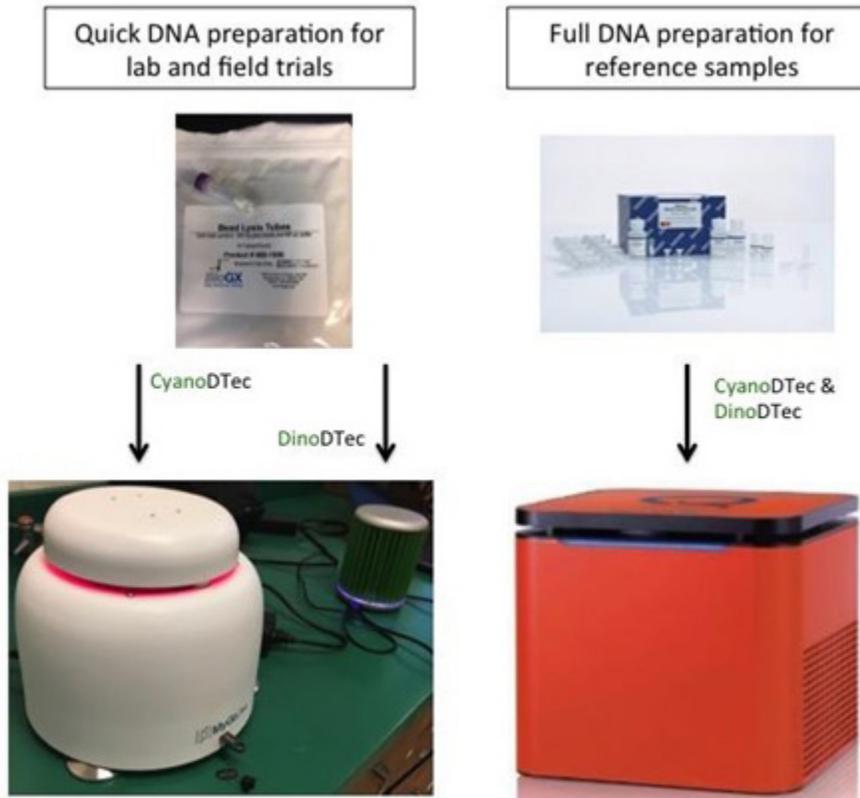


Figure 2. Quick DNA extractions for lab and field trials were performed with bead lysis tubes and qPCR was carried out with the CyanoDTec or DinoDTec kit on the MyGo Pro[®] or MyGo Mini[®], respectively. Full DNA extractions for reference samples were performed with the QIAgen DNeasy Blood and Tissue Kit and both qPCR assays (CyanoDTec and DinoDTec) were performed on the QuantaBio Q qPCR machine.

Phytoxigene's DinoDTec kit uses qPCR to measure presence of the *sxtA* gene sequence essential for the production of saxitoxin in dinoflagellates. The presence of this gene is indicative of the potential for saxitoxin production and risk of Paralytic Shellfish Poisoning. The relative increase of the amount of these genes has been shown to correlate with increased levels of toxins both in the environment and in the shellfish feeding within these environments. Results are commonly expressed in gene copies per liter. The DinoDTec assay is packaged in an aluminum foil pouch. Each test includes an internal control target that measures for inhibition within the assay. This Internal Amplification Control (IAC) amplifies independent of whether the target toxin gene is present or not, and serves to validate the amplification process and result. A set of nucleic acid standards (100, 1,000, 10,000, 100,000 and 1,000,000 copies per reaction) is available from Phytoxigene for creating a standard curve for calculating copy number in unknown samples.

The limit of quantification for the *stxA* assay, with a 95% degree of confidence is 25 copies per reaction. Samples that are positive below this level may be reported and read as such by most PCR machine software. Levels as low as 10 copies per reaction have shown reasonable levels of reproducibility and accuracy and reflect a C_q value (when fluorescence crosses background threshold) of approximately 3 cycles below the 100 gene copy standard. Caution should be made when interpreting results where the C_q values are more than 5 cycles below the lowest standard. These values reflect theoretical levels as low as one gene copy per reaction. Any samples that return a C_q that is 3 or more cycles above the lowest standard should be reported as BDL. Samples that do not amplify after 40 cycles should be considered as not detectable. The starting volume of the primary sample will have considerable impact on the overall limit of detection of the assay when reporting copy number per liter and relating this to an estimate of cell number. *SxtA* copy number variability within algal cells (Murray et al., 2011; Stüken et al., 2011; Stüken et al., 2015) can complicate cell estimation. A number of studies have however shown that *sxtA* gene copy correlates with toxin concentration, inferring that *stxA* gene copy number may be more relevant for assessment of toxin risk and production. The extraction protocol involves concentrating samples onto filters and bead beating for 5 minutes using commercially available bead lysis tubes (BioGX, Birmingham, Alabama, USA). The resulting lysate is transferred to a sterile Eppendorf tube and stored on wet ice until qPCR set up. The lyophilized qPCR mix is reconstituted in 80µL sterile molecular biology grade water, and for reaction set-up 20µL of that mix is combined with 5µL of the template. Any qPCR machine may be utilized with the kit, provided it contains two separate channels for excitation/detection of the following two fluorophores: FAM (495/516nm) and Cy3/CalFluor Orange (538/559nm). The portable MyGo Mini® (IT-IS Life Science Ltd.) was used for all DinoDTec lab and field tests for this demonstration (Figure 2). The qPCR used for all corresponding reference samples was a QuantaBio Q qPCR machine as described below in the Reference section.

PERFORMANCE EVALUATION 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 few 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 or species in freshwater and marine systems.

ACT conducted two laboratory tests and four field tests as part of the toxin/HAB species evaluation. One of the lab tests focused on freshwater species and associated toxins, and the second evaluated marine species and associated toxins. 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 instrumentation/kits. 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 conducted 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 test kits compared to reference sample analysis which included independent analysis of toxin concentrations using USEPA adopted ELISA methods and LCMS measurements conducted by Dr. Raphe Kudela using state of California certified protocols. In addition, independent reference qPCR using a full DNA extraction protocol (versus bead beating) and microscopic counts of targeted HAB species were conducted by ACT personnel during each lab test.

The freshwater lab testing took place at BGSU from July 11-15, 2018. The testing involved genetic material extracted from freshwater HAB cultures including 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 took place at MLML from September 11 – 14, 2018 and October 1 – 3, 2018. The testing utilized two UCSC cultures for saxitoxin (putatively *Alexandrium catenella* that exhibit different toxin profiles, UCSC pers. comm.). Cultures were maintained in L1 growth media at 15°C under a 14:10 h light:dark photoperiod in an environmental chamber illuminated at 142 μmol (photons) $\text{m}^{-2} \text{s}^{-1}$ with standard F40 cool white fluorescent tubes. 0.2 micron filtered Monterey Bay seawater was utilized for culture propagation and dilutions. Experiments were conducted with the cultures individually (with a target of 10 cells in the reaction) as well as mixed together (0, 5, 50, 200, or 500 cells per mL).

Field Tests

A rigorous field testing program was designed to provide a wide variety of toxin-producing species within various freshwater and marine ecosystems. The field tests provided a natural range of test conditions, including 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 sites to provide the greatest variation in test conditions. Freshwater test sites included two locations within the Great Lakes (western Lake Erie and Sandusky Bay), as well as Pinto Lake (Watsonville, CA) and an additional inland hot spot near Monterey, CA. Saltwater test sites were conducted in both the northern Atlantic and the mid Pacific. Test sites on the east coast were selected based on current monitoring sites within Long Island Sound established by Dr. Chris Gobler (SUNY). The west coast site (Monterey Municipal wharf) was selected based on current monitoring efforts by Dr. Smith.

Reference Sample Collection and Analytical Methods

Reference samples were collected during all field and laboratory tests for direct comparison between test kits and independently analyzed laboratory results. All samples were processed to analyze toxin concentrations, toxin-producing genes (using a full DNA extraction protocol), and phytoplankton abundance. Toxin concentrations were determined using liquid chromatography/mass spectrometry (LCMS-LR) and enzyme-linked immunosorbent assays (ELISA). Reference sample LCMS measurements were conducted at the lab of Dr. Raphe Kudela using state of California certified protocols. Reference sample ELISA measurements were conducted by ACT personnel at the host lab site: University of Michigan using EPA Method 546 and the Abraxis kit (catalog #520011) for cyanotoxins, and SUNY and MLML using the BIOO Scientific Saxitoxin (PSP) ELISA Test Kit (catalog #1034) for dinoflagellate saxitoxins. In addition, independent qPCR and microscopic counts of targeted HAB species were conducted by ACT personnel for all reference samples. Method details are described below. Every test site also conducted one blank spike in clean distilled water.

Liquid Chromatography/Mass Spectrometry

Samples for cyanobacterial 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 -80°C. The whole water fraction was analyzed from a 50 mL sample poured into amber glass bottles and stored at -80°C. 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 -80°C 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 relative cross-reactivities of the Abraxis Kit antibodies to microcystin congeners.

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

Enzyme-Linked Immunosorbent Assays (ELISA)

Samples for toxin analysis by ELISA were collected for both whole water and the dissolved fraction. The dissolved fraction was analyzed from a 10 mL sample (100mL for *Alexandrium* laboratory tests; 5mL for MLML field testing sites) filtered through 0.2 µm nylon filters into amber glass vials and stored at -80°C. The whole water fraction was analyzed from 50 mL samples

(20mL – 50mL for MLML field testing sites) poured into an amber glass bottle stored at -80°C. 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 was performed by ACT staff at the local test-site laboratory (with the exception of MLML samples which were run at UCSC).

Samples for particulate (intracellular) saxitoxin analysis by ELISA were produced in triplicate by filtering a required volume of sample water through a 25 mm, 10 µm Millipore/Merck Isopore filter (TCTP02500). The volume filtered was determined from previous monitoring results (up to 2 L per filter) to try to ensure reaching detection levels. Two replicates were analyzed for each reference sample and one saved as a back-up.

ELISA analysis for microcystins was performed according to EPA 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. The BIOO Scientific Saxitoxin (PSP) ELISA Test Kit (catalog #1034) was used to analyze dinoflagellate saxitoxins in lab tests with *Alexandrium* sp. and field samples.

Quantitative Polymerase Chain Reaction (qPCR)

For quantitative polymerase chain reaction (qPCR), three replicates were filtered using a 25 mm, 10 µm Millipore/Merck Isopore filter (TCTP02500) for the saltwater collections. For freshwater test sites for cyanobacteria a 25 mm, 2 µm pore size filter was used for the triplicate collections. For all MLML lab and field tests, 25 mm 0.65 µm Millipore Durapore® (DVPP02500) were used. Filters were stored in 2 mL polypropylene Eppendorf tubes and kept on ice until storage in a -80°C freezer. At the end of sample collection, two of the filters were extracted and analyzed and one was retained as a hold-back for reanalysis if needed. Each extract was sub-sampled into two wells on the qPCR plate for analytical duplicates.

The reference qPCR filters from each site/date were thawed and extracted with the QIAgen DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). 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 56°C 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). Total final elution volume was 60µL. Reactions were run on a QuantaBio Q qPCR machine (Figure 2, right panel; <https://www.quantabio.com/>) with the same cycling parameters as outlined for the MyGo Mini which was used for the saltwater field samples. Cycling on both machines was per manufacturer's directions: initial denaturation step of 120 sec at 95°C followed by 40 cycles of 10 sec at 95°C (3°C per second ramp rate) and 45 sec at 64°C (1.5°C per second ramp rate). Copy numbers per reaction were calculated by the software comparing Cq's to those of a full standard curve that covered five orders of magnitude (1x10² – 1x10⁶ copies per reaction; proprietary Phytotoxigene™ components) generated prior to the start of the field campaign and imported for each analysis. If the Cq for the internal control within each reaction (IAC) was greater than 1.5 cycles above 31, it was considered inhibited and the sample was diluted, re-run, and gene copies recalculated.

Cell Counts

Phytoplankton cell abundances were quantified for each reference sample to determine relative abundance of target species. Saltwater sample counts focused on dinoflagellates and diatoms, while freshwater sample counts focused on cyanobacteria. For the cell counts, whole water samples were fixed with acidified Lugol's for a final preservative concentration of 4% (v/v). Cell abundance of target species was enumerated microscopically 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 Testing (CyanodTec)

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 *Microcystis* (2386), and a cylindrospermopsin producing culture of *Cylindrospermopsis raciborskii* (CS-506). *M. aeruginosa* LE3 and 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 the start of experiments.

Common Lysate Trial

The first laboratory trial consisted of analyzing the diluted cultures of the toxin producing LE3 used to make a common lysate for toxin analysis using the U.S. EPA freeze-thaw Method 546. 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]; Table 1). Microscopy results of the LE3 samples post-testing indicated that the culture was not pure and that a significant amount of *Planktothrix* (also a microcystin producer and detectable with the CyanoDTec kit) 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 of culture (mLs) 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 ID	mLs LE3	<i>Microcystis</i> cells/mL	<i>Planktothrix</i> cells/mL	<i>Cylindrospermopsis</i> 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

Results for 16S gene copy abundance from both qPCR platforms were in the same order of magnitude (Figure 3), but were an order of magnitude higher than estimates of cyanobacteria cell densities by microscopic counts which is somewhat typical for non-axenic cultures. Reference qPCR for *mcyE/ndaF* detection on the QuantBio Q platform was lower for both cell concentrations as compared to the samples run on the MyGo Pro which likely reflects loss of genetic material during the column-based, multi-step extraction process. In fact, toxin gene abundance results from both platforms were lower than cell counts, which supports the loss of material during both types of extraction protocols. 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.

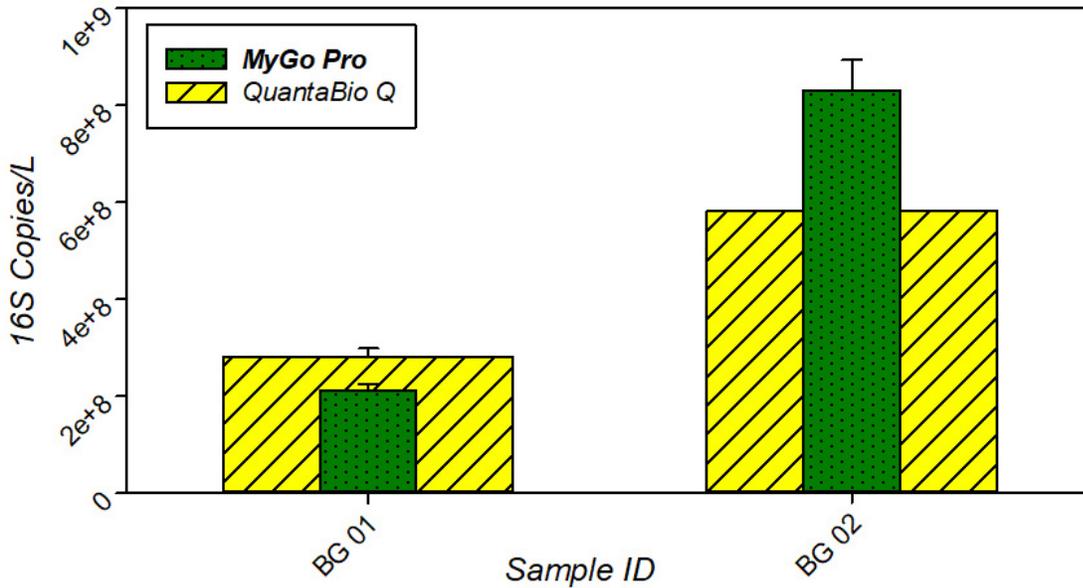


Figure 3. Estimates of cell density in 16S gene copies (total cyanobacteria) per liter derived from the QuantaBio Q reference qPCR using the CyanoDTec kit (yellow bars) and MyGo Pro using the CyanoDTec kit (green bars). Error bars represent one standard deviation of the average for the two sample replicates and the two analytical replicates of a given sample extract (n = 4).

Toxin concentrations (Figure 4, bottom panel) support the increase in cell concentrations and toxin gene abundances measured on both platforms. Only a single sample was produced for each toxin concentration – results plotted in Figure 4 are from triplicate analyses.

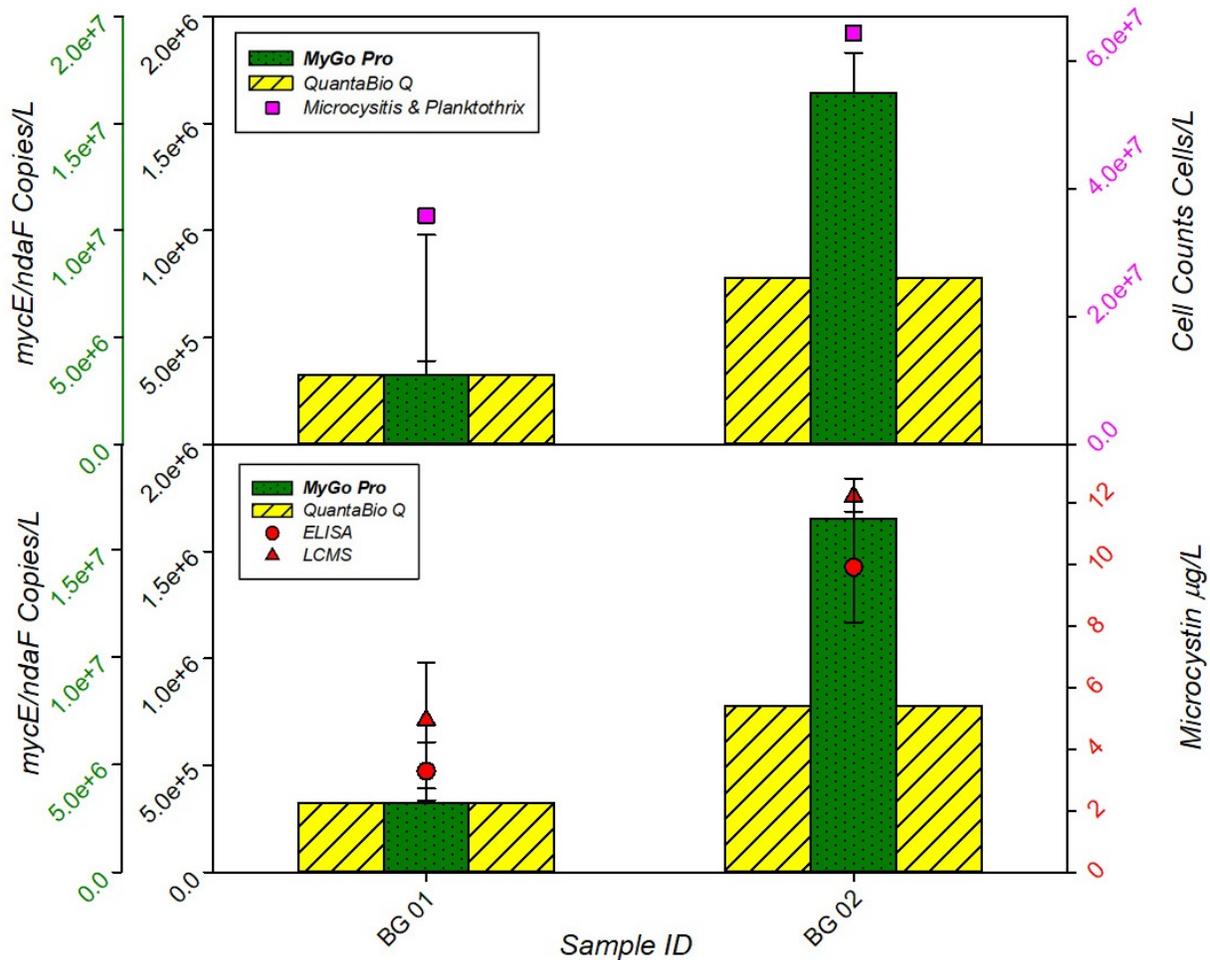


Figure 4. *Top Panel:* Correspondence of MyGo Pro (outer left, green axis, green bars) and QuantaBio Q (inner left, black axis, yellow bars) measures of toxin gene abundance mycE/ndaF (microcystin + nodularin) per liter. Cell counts for *Microcystis* + *Planktothrix* (pink squares) are plotted on the secondary y-axis. *Bottom Panel:* Correspondence of MyGo Pro (outer left, green axis, green bars) and QuantaBio Q (inner left, black axis, yellow bars) measures of toxin gene abundance (mycE/ndaF) with microcystin concentration measured via ELISA (circles) and LCMS (triangles) plotted on the secondary y-axis.

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 (inconsistently identified during counting).

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	<i>Microcystis</i> (LE3) 10 ³ Cells/L <i>mcyE</i> ⁺	<i>Planktothrix</i> ¹ 10 ³ Cells/L <i>mcyE</i> ⁺	<i>Cylindrospermopsis</i> (CS-506) 10 ³ Cells/L <i>cyrA</i> ⁺	Total 10 ³ Cells/L
BG 04	1.0	-	24,677	38,057	-	62,734
BG 05	-	1.0	-	-	19,270	19,270
BG 06	1.0	1.0	24,769	5,690	19,239	49,698
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

¹A contaminant of the LE3 culture.

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 toxin genes. qPCR results for 16S for both platforms are depicted in Figure 5 and are several orders of magnitude higher than cell counts (Table 2) likely representative of the non-axenic nature of the cultures used. Except for sample BG-04, gene target abundances generally follow the increasing cell concentrations over both days. Reference qPCR (QuantaBio Q) results were consistently lower, but in the same order of magnitude, as compared to the MyGo Pro samples that did not go through the full extraction protocol.

qPCR results for *mcyE/ndaF* and *cyrA* gene markers (Figure 6) confer with representative control samples of *Microcystis* only (BG-04), *Cylindrospermopsis* only (BG-05), and a mixture of the two (BG-06). Signal inhibition for *mcyE/ndaF* seen in BG-04 and BG-06 may be an artifact of both extraction protocols being inhibited by high cell abundances (i.e. inability to capture all genetic material, note cell numbers in Table 2 are reported as 10³). Overall, all data (qPCR, toxin, cell counts) for the day two sample set (BG-18, 19, 20) trend according to the experimental design of decreasing *Microcystis* and increasing *Cylindrospermopsis* (Figure 6). However, the same pattern can be seen as outlined in the experiment above: qPCR signal from the reference samples (QuantaBio) is lower than test samples on the MyGo Pro (note different scales on y-axis in Figure 6), and both are lower than the number of cells counted. Data are also presented as cross-plots of cell counts (Figure 7; *Microcystis* + *Planktothrix*) and toxin analyses (Figure 8) against *mcyE/ndaF*

qPCR on both platforms. *Cylindrospermopsis* cell counts are plotted against *cyrA* qPCR on both platforms (Figure 9).

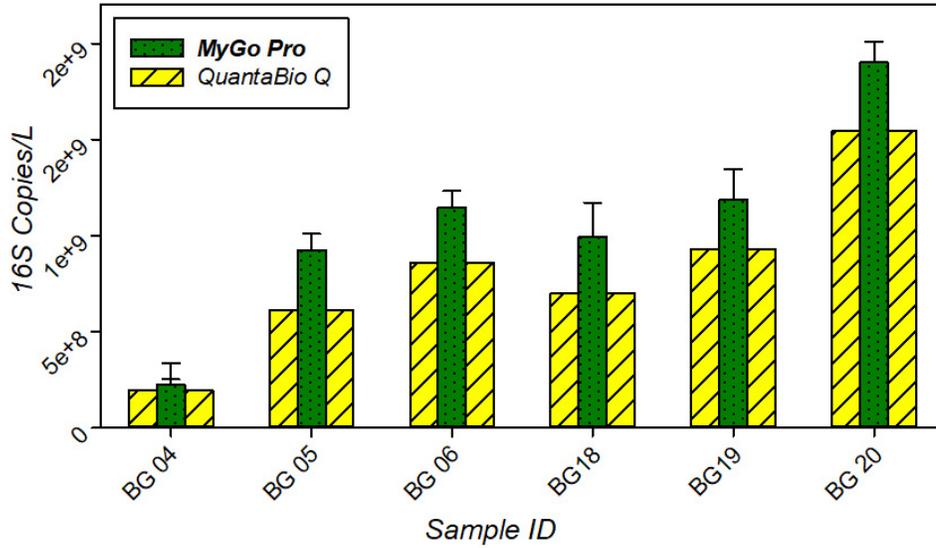


Figure 5. Mixed Species Trial qPCR results for 16S. MyGo Pro for test samples (field based extraction protocol) represented by green bars and QuantaBio Q for reference samples (full extraction protocol) represented by yellow.

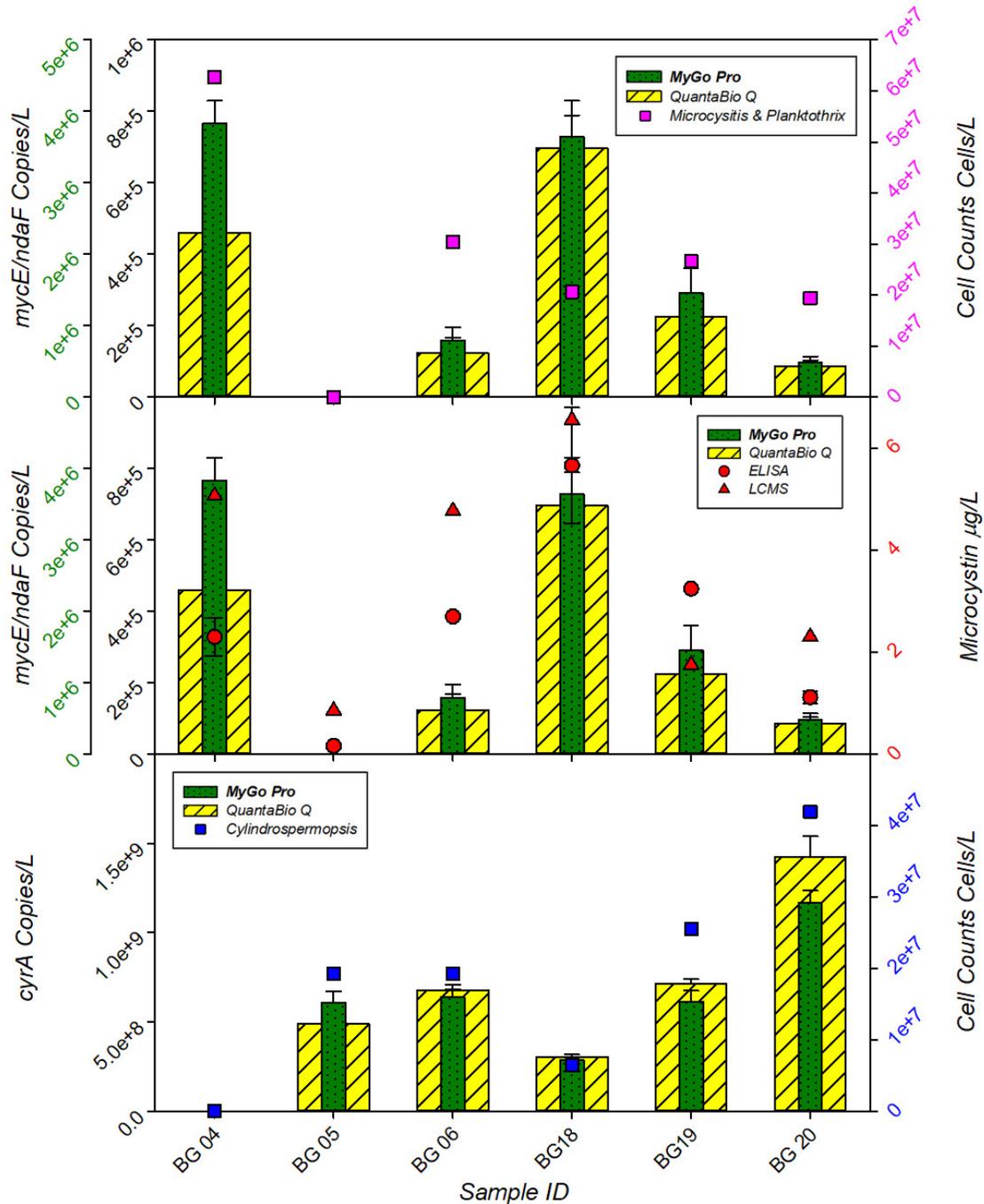


Figure 6. Results of the BGSU mixed species trial using *Microcystis* (LE3) and *Cylindrospermopsis* (CS-506.) *Top Panel:* Comparison of toxin gene concentrations (mcyE/ndaF) estimated by MyGo Pro (outer left axis, green bars) and QuantaBio Q (inner left axis, yellow bars) to cell counts shown on the secondary y axis for *Microcystis* and *Planktothrix* (pink squares.) *Middle Panel:* Comparison of gene concentrations (mcyE/ndaF) to microcystin concentrations estimated by ELISA (red circles) and LCMS (red triangles). *Bottom Panel:* Comparison of toxin gene concentrations (cyrA) to cell counts for *Cylindrospermopsis* (blue squares). Error bars are one standard deviation (n = 2) for ELISA and (n=4) qPCR; LCMS-LR have no error bars as there was a single value. We note the *Microcystis* culture was contaminated with *Planktothrix*.

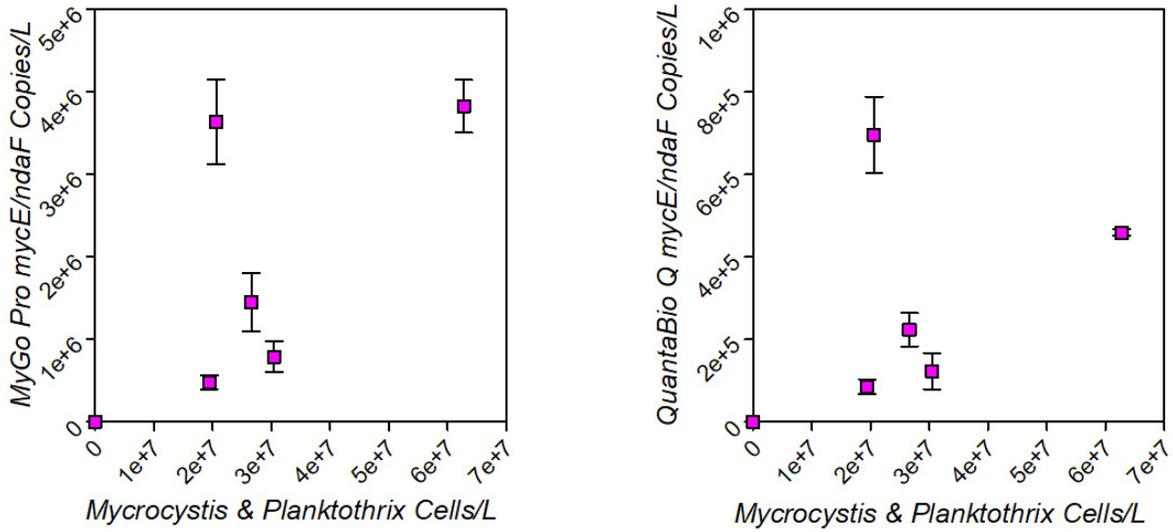


Figure 7. Cross plot of cell counts (*Microcystis* and *Planktothrix*) against MyGo Pro (left) and QuantaBio Q (right) *mycE/ndaF* copies/L.

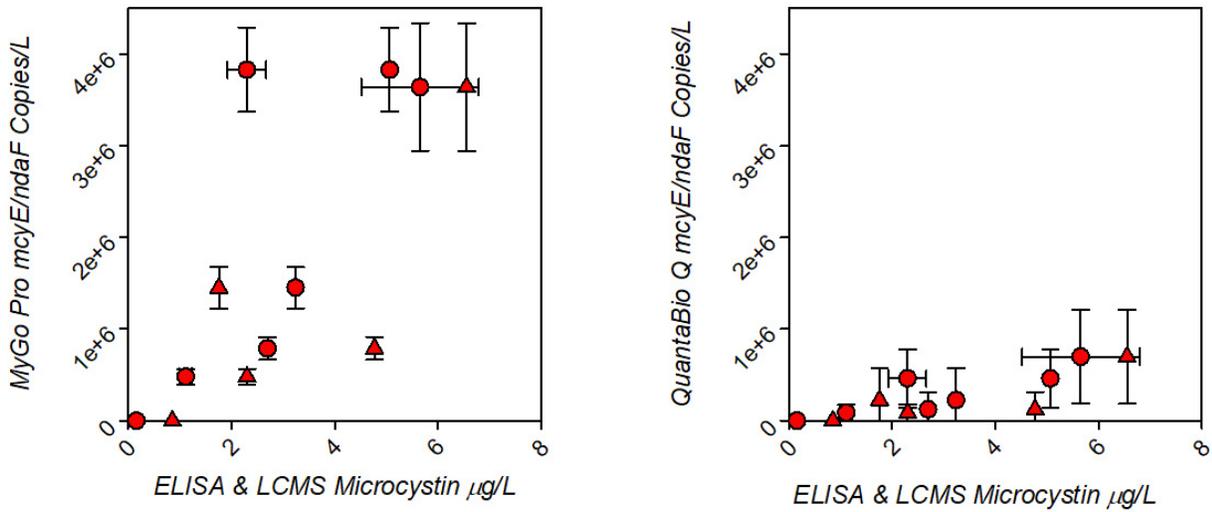


Figure 8. Cross plot of toxin analysis results (LCMS-LR are triangles; ELISA are circles) against MyGo Pro (left) and QuantaBio Q (right) *mycE/ndaF* copies/L.

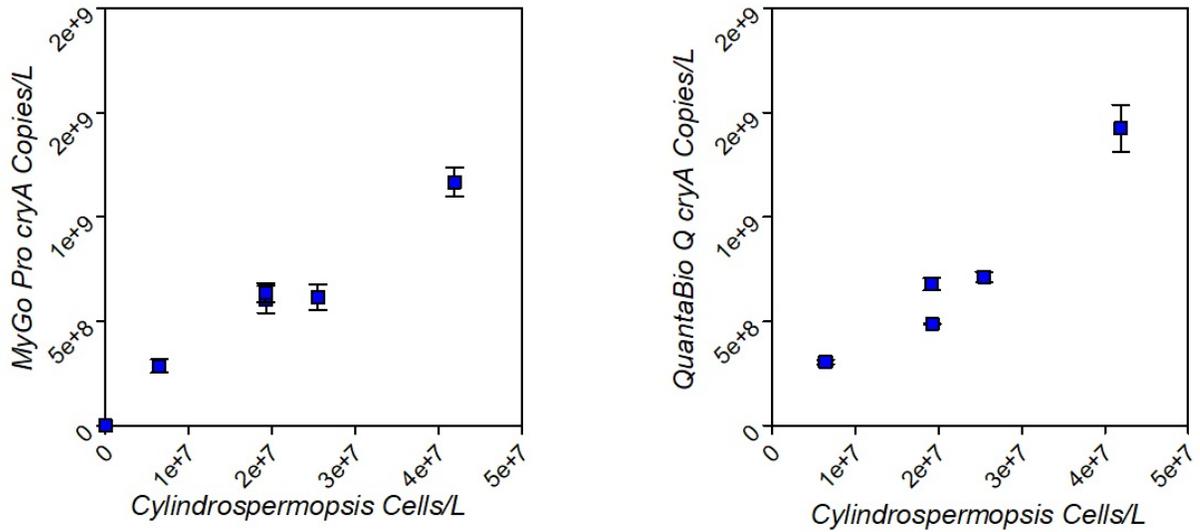


Figure 9. Cross plot of cell counts of *Cylindrospermopsis* cells/L and MyGo Pro (left) and QuantaBio Q (right) *cyrA* copies/L.

Range Trial

The laboratory range trial consisted of mixtures of both toxic *Microcystis* (LE3) and non-toxic *Microcystis* (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). As outlined above, culture LE3 was contaminated with *Planktothrix* and the latter was inconsistently identified during counting since it was not an original target for the experiment. 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 2386	<i>Microcystis</i> (LE3 & 2386) 10 ³ Cells/L	<i>Planktothrix</i> 10 ³ Cells/L	Total 10 ³ Cells/L
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 16S copies for the range trial samples (MyGo Pro versus reference samples on the QuantaBio Q) are shown in Figure 10. A total of 16 mLs of culture were added to each sample (media background), with the proportion of toxic and non-toxic strains of *Microcystis* varied as shown in Table 3. The 16S gene marker copies for both platforms (Figure 10) were

relatively consistent across the mixtures (a factor of two), but somewhat inconsistent with the microscopic cell count data (Table 3). This variability may be an artifact of both extraction protocols being variably inhibited by high cell abundances (i.e. inability to capture all genetic material, note cell numbers in Table 3 are reported as 10^3). The *mcyE/ndaF* qPCR signal from the quick extraction samples run on the MyGo Pro followed a less variable trend with increasing toxicity (as confirmed by toxin analyses) than the signal from the reference samples on the QuantaBio Q platform (which tended towards a stronger uncoupling with increased dosing of toxin gene to culture mixes). Data are also presented as cross-plots of cell counts (Figure 12; *Microcystis* + *Planktothrix*) and toxin analyses (Figure 13) against *mcyE/ndaF* qPCR on both platforms.

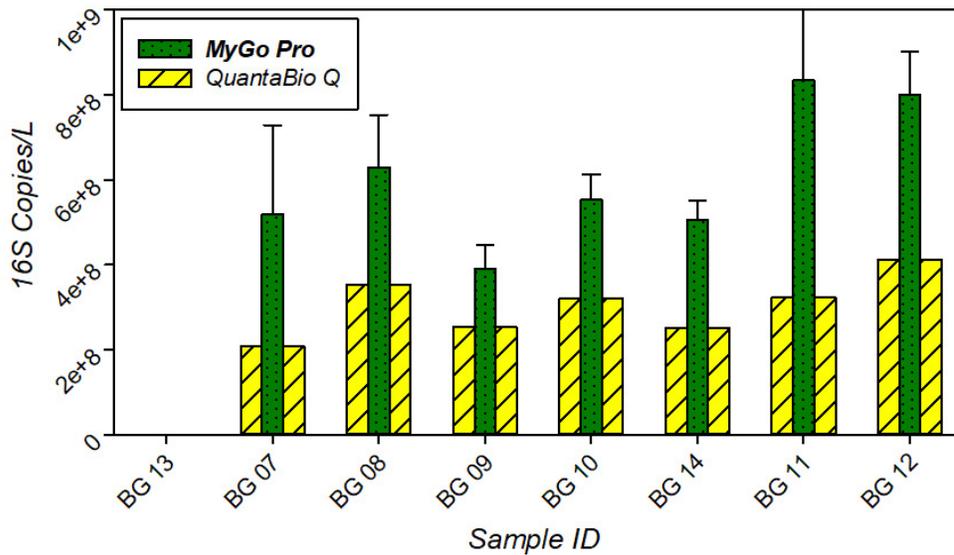


Figure 10. Comparison of 16S gene copies per liter from the BGSU Range Trial by MyGo Pro (green bars) and QuantaBio Q (yellow). BG 13 was a media blank.

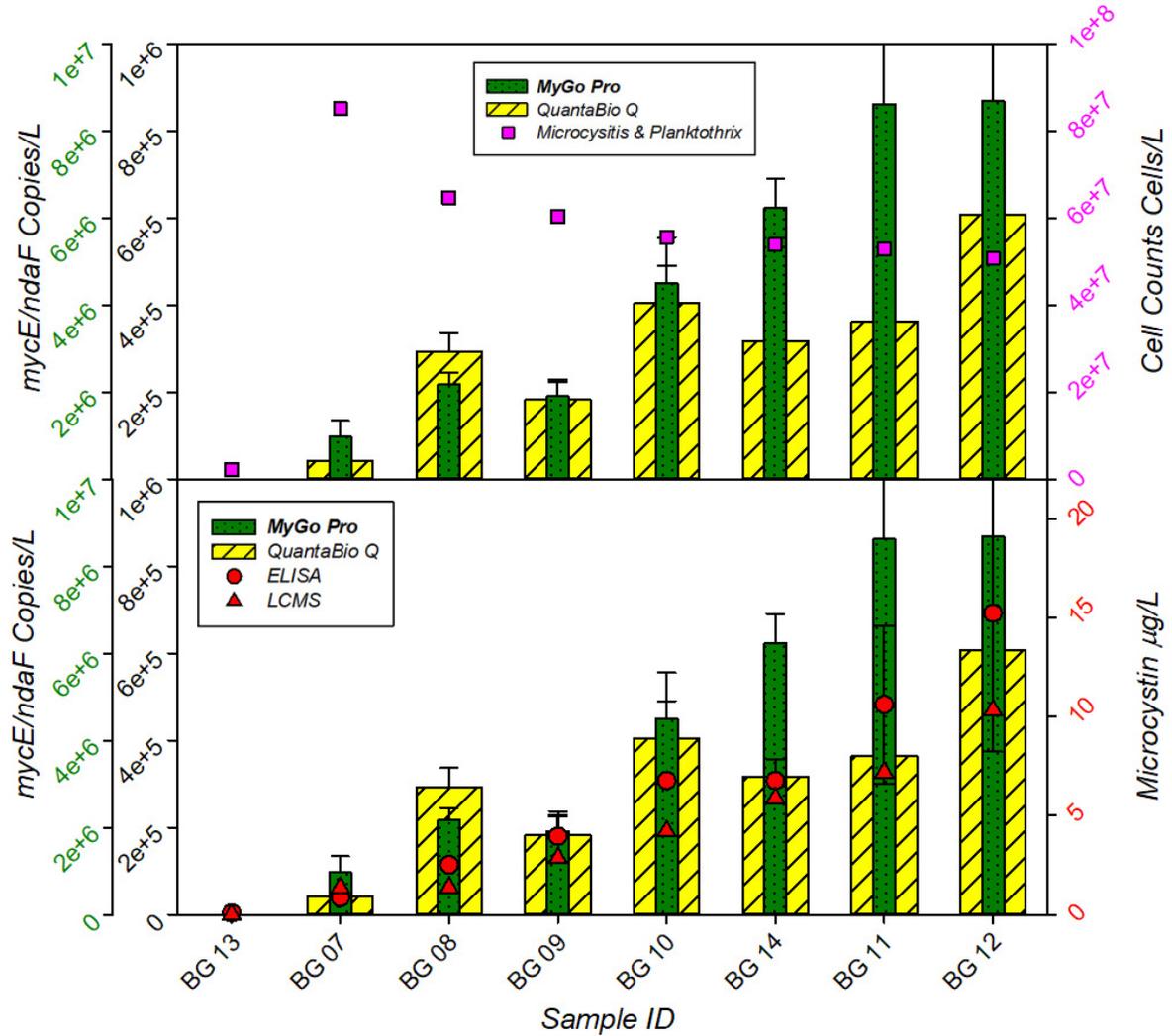


Figure 11. Results for the BGSU range trial. Samples BG10 and BG14 are duplicates in terms of the culture mixtures but were made independently. Sample BG13 was a media blank with no culture added. *Top Panel:* Comparison of toxin gene concentrations (mycE/ndaF) estimated by MyGo Pro (field based extraction protocol, green bars, left outer axis), mycE/ndaF estimated by QuantaBio Q (full extraction protocol, yellow bars, left, inner axis) and *Microcystis* and *Planktothrix* counts in cells/L (pink squares, right axis). *Bottom Panel:* Comparison of toxin gene concentrations (mycE/ndaF) estimated by MyGo Pro (outer left axis, green bars), QuantaBio (inner left axis, yellow bars) with ELISA (red circles, right axis) and LCMS (red triangles, right axis) estimates of microcystin. Estimates of 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). We note that the *Microcystis* cultures were contaminated with *Planktothrix*.

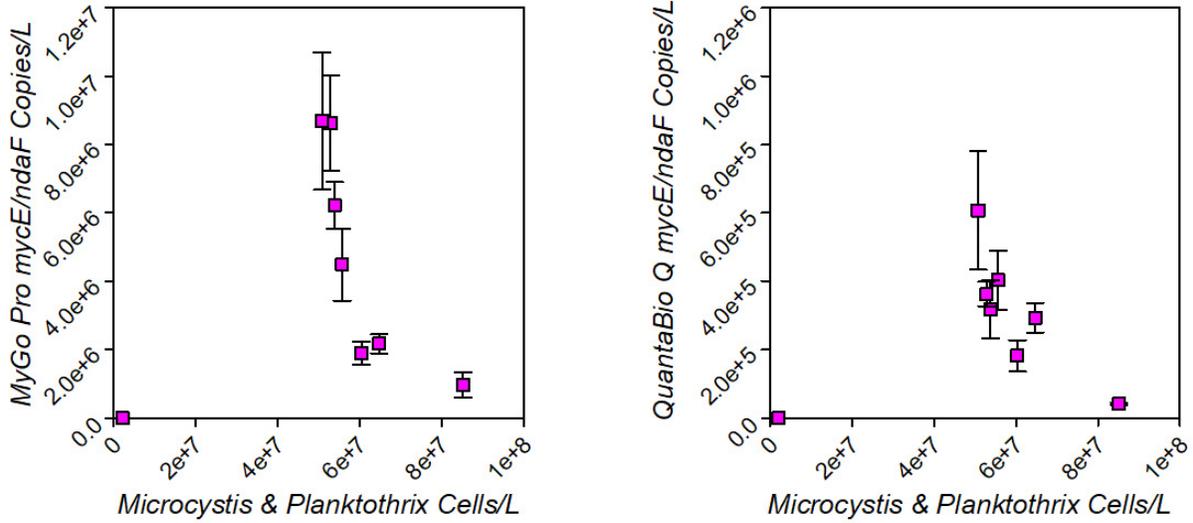


Figure 12. Cross plot of cell counts cells/L (*Microcystis and Planktothrix*) against MyGo Pro (left) and QuantaBio Q (right) mycE/ndaF copies/L.

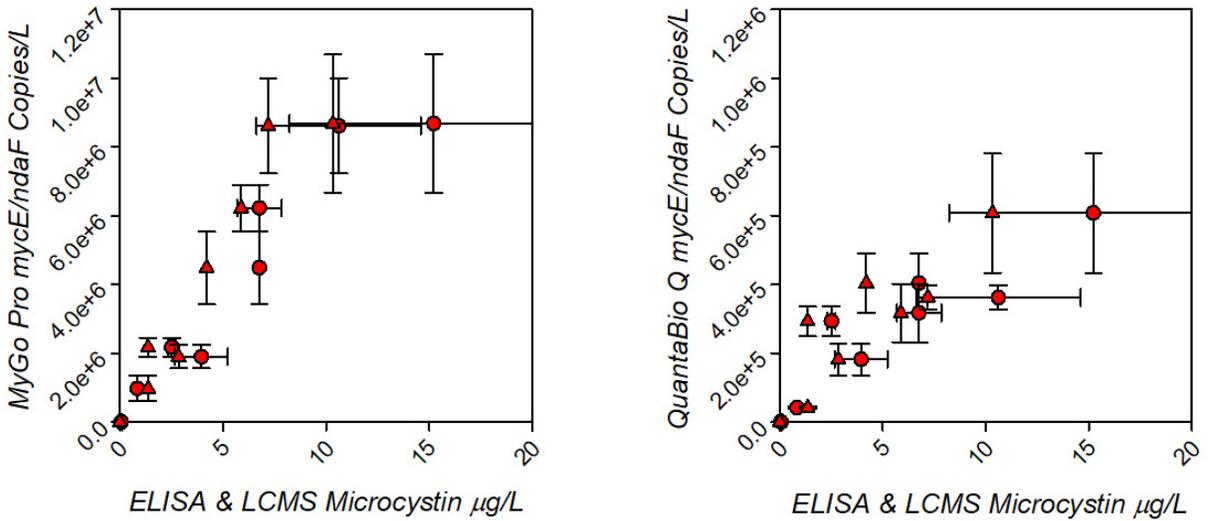


Figure 13. Cross plot of toxin analysis results (LCMS-LR are triangles; ELISA are circles) against MyGo Pro (left) and QuantaBio Q (right) mycE/ndaF copies/L.

Marine Lab Testing (DinoDTec)

The saltwater lab testing took place at Moss Landing Marine Labs (California) from September 11 – 14, 2018 and October 1 – 3, 2018. The testing utilized two UCSC cultures for saxitoxin (putatively *Alexandrium catenella* [AC-17, AC-18] that exhibit different toxin profiles, USCS pers. comm.). Cultures were maintained in L1 at 15°C under a 14:10 h light:dark photoperiod in an environmental chamber illuminated at 142 μmol (photons) $\text{m}^{-2} \text{s}^{-1}$ with standard F40 cool white fluorescent tubes. 0.2 micron filtered Monterey Bay seawater was utilized for culture propagation and dilutions. During the saltwater lab testing, a total of seven samples were generated. Filtration and collection of supporting reference samples were all conducted immediately following culture manipulations (dilutions, mixing, spiking).

Table 4. MLML Lab sample concentrations.

Sample ID	Target Cell Density (Cells/L)	Volume Filtered (mL)	Observed <i>Alexandrium</i> Cell Counts Cells/L
ML 02	Stock AC17	200	46,000 \pm 6,702
ML 03	Stock AC18	200	41,000 \pm 9,055
ML 09	0	100	0
ML 10	5,000	100	14,000 \pm 957
ML 11	50,000	100	38,000 \pm 3,916
ML 12	200,000	100	148,000 \pm 4,000
ML 13	500,000	100	477,000 \pm 2,121

The two *A. catenella* cultures were tested individually as well as mixed together (Table 4). There was agreement between sxtA qPCR signal from the MyGo Mini and QuantaBio Q (reference samples) for the sxtA gene in the dilution series (Figure 14). The BDL result for sample ML-10 (14,000 cells/L) represented 17.5 cells and 117 cells in the reaction for the MyGo Pro and QuantaBio Q platforms, respectively. Saxitoxin concentrations based on ELISA followed the increasing cell concentrations where the gene target was detectable (Figure 14; ML-11, 12 and 13). Note the order of magnitude difference in qPCR concentrations on primary y-axes for Figure 14, reflecting the likely loss in genetic material during the full extraction procedure used for reference samples (QuantaBio Q). Data are also presented as cross-plots of cell counts (Figure 15; *Alexandrium*) and toxin analysis (Figure 16) against sxtA qPCR on both platforms.

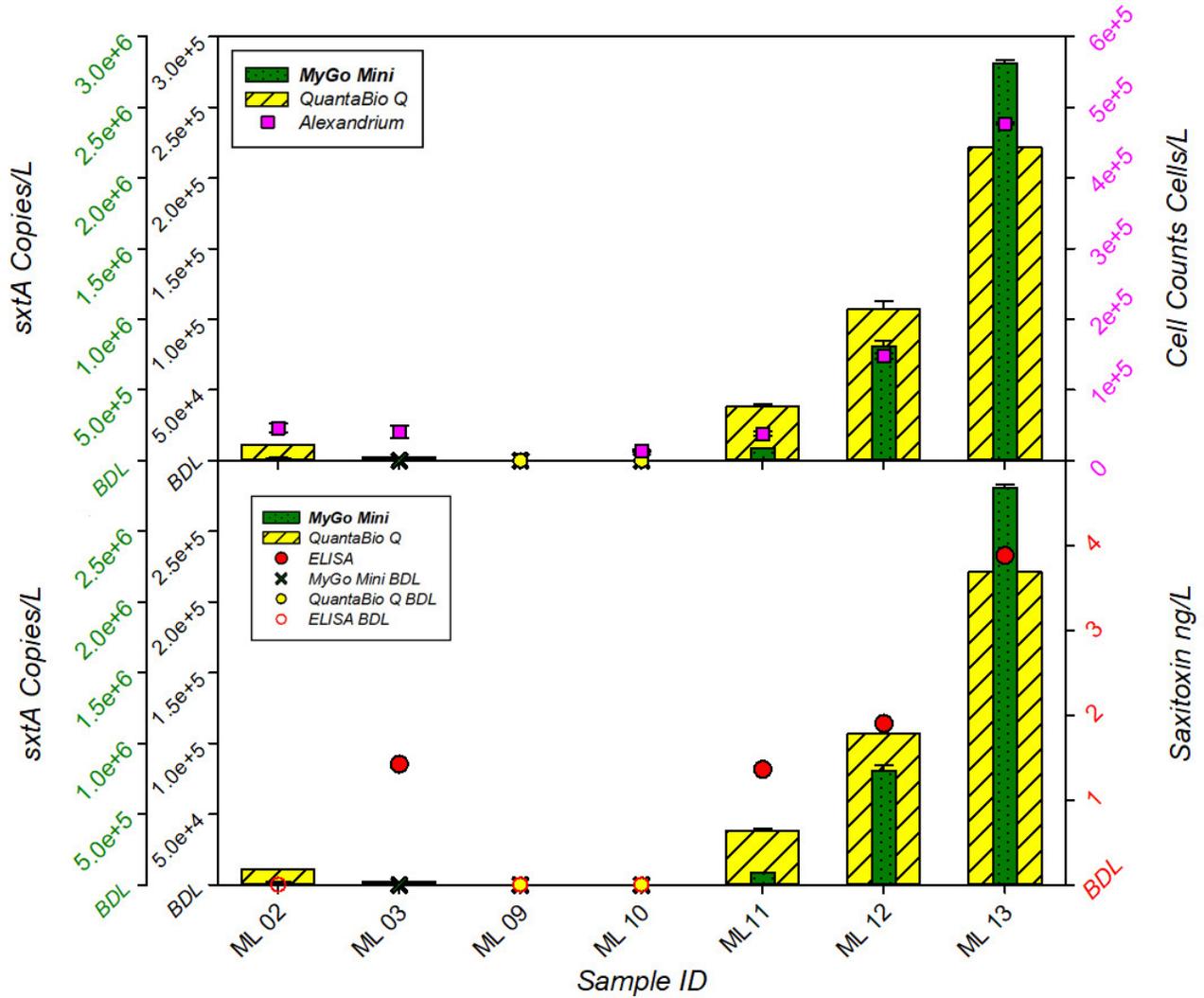


Figure 14. *Top Panel:* Comparison of sxtA qPCR detection from the MyGo Mini (green bars, outer left axis) and QuantaBio Q (yellow bars, inner left axis) and cell counts of *Alexandrium catenella* (pink squares, right axis) for the dilution series outlined in Table 4. *Bottom Panel:* Comparison of sxtA qPCR detection from the MyGo Mini (green bars, outer left axis) and QuantaBio Q (yellow bars, inner left axis) and ELISA saxitoxin estimates (red circles, right axis). BDL = Below Detection Limit. Error bars represent one standard deviation of the average of two analytical reps from each of two filter replicates (n=4).

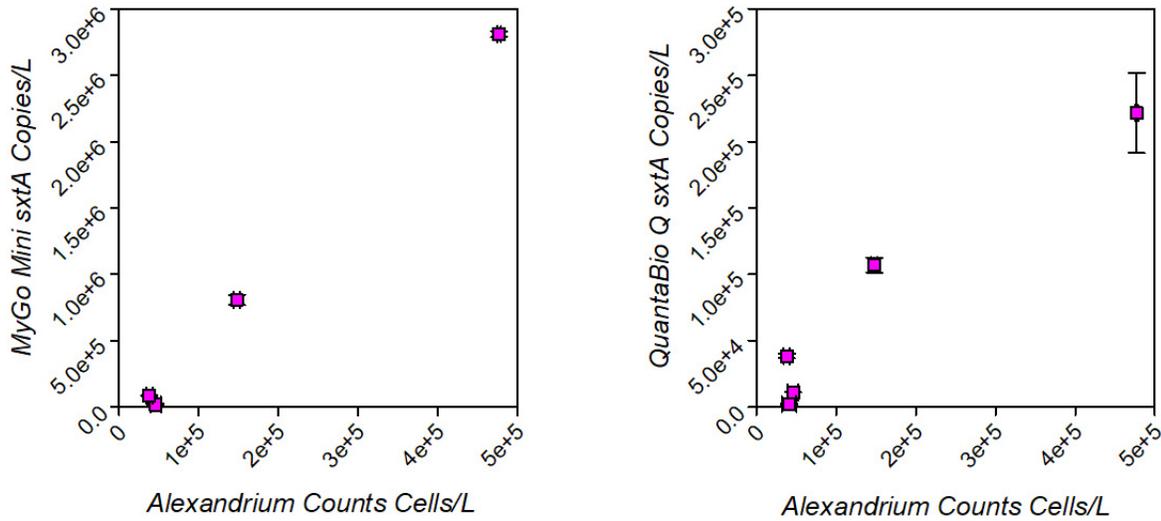


Figure 15. Cross plots of estimates of sxtA by MyGo Mini (left) and QuantaBio Q (right) and *Alexandrium* cell counts.

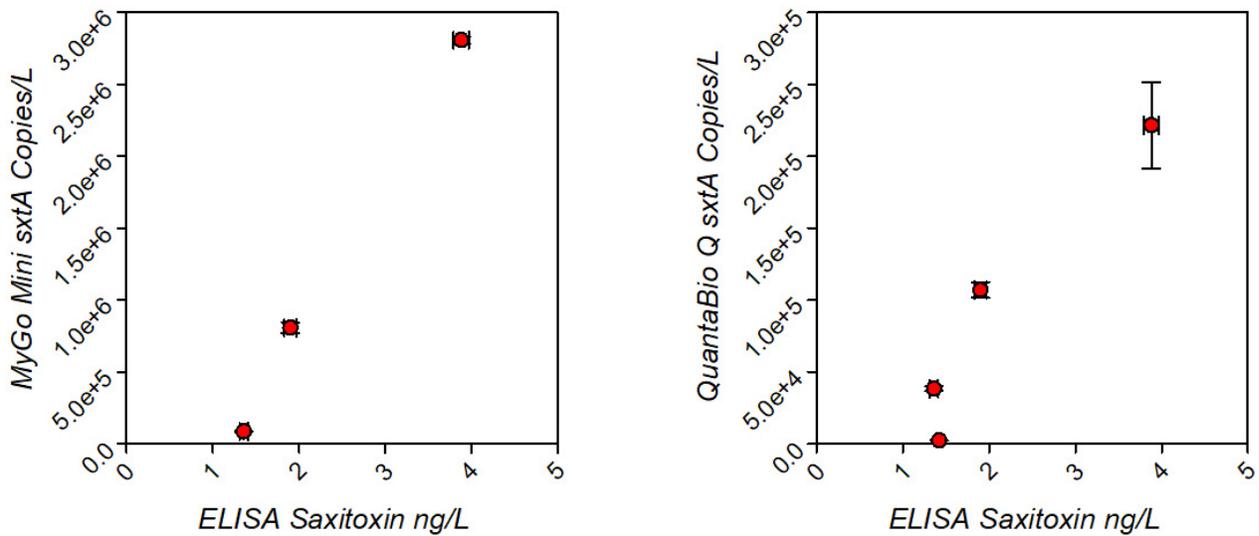


Figure 16. Cross plots of estimates of sxtA copy numbers by MyGo Mini (left) and QuantaBio Q (right) and saxitoxin estimates.

RESULTS OF FIELD TESTS

Field tests in three geographically distinct locations were conducted as part of the performance evaluation of the CyanoDTec and DinoDTec kits: Long Island NY, Lake Erie MI (Western Lake Erie and Sandusky Bay), and Pinto Lake near Monterey Bay CA. The Western Lake Erie and Sandusky Bay locations were both sampled on two different occasions to capture a greater dynamic range in HAB 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.

Long Island Sound, New York

The Phytoxigene™ DinoDTec kit was operated for three consecutive days (May 7 - 9, 2018) at three fixed dockside stations on Long Island, NY (Figure 17, left): Meetinghouse and James Creeks (both adjacent to the Peconic River) and Weesuck Creek (a tributary of Shinnecock Bay). These stations are sampled weekly by Dr. Christopher Gobler's laboratory (Stony Brook University) as part of the New York's Department of Environmental Conservation shellfish monitoring program. This region has a history of toxic *Alexandrium* spp. blooms and in fact, during the ACT field campaign, shellfish closures were put into place for western Shinnecock Bay due to elevated toxin and cells (<https://ioos.noaa.gov/news/act-evaluates-new-portable-hab-sensors/>). Whole surface water samples were collected via a bucket and poured through a 150 micron screen into a carboy to exclude large zooplankton and detritus. For qPCR samples, either 2L (for reference qPCR) or 3L (for on-site qPCR) were collected from the well-mixed carboy and poured through a PVC cell concentrating device to capture particles ≥ 11 microns (Figure 17, right). For reference qPCR filters (n=3), material retained on the 11 micron mesh was back-flushed using filtered seawater (FSW) into the cup of a filtration rig and concentrated (5 mmHg vacuum) onto a 10 micron Durapore® filter (PVDF; EMD Millipore, Billerica, MA, USA). Filters were aseptically transferred (sample side facing inward) via forceps to individual Eppendorf tubes, transported on wet ice back to the laboratory where they were moved to -80°C for storage until analysis.

For on-site qPCR samples, material retained on the 11 micron mesh was back-flushed using filtered seawater (FSW) into a tri-pour beaker, and a syringe filter was used to concentrate material onto a 10 micron filter. The filter was aseptically transferred (sample side facing inward) via forceps to BioGx bead lysis tubes. Cells were disrupted by vortexing using a Scientific Industries Vortex Genie 2 on high (setting 10 on the dial) for 5 min. Tubes were then spun for 30 sec in a VWR mini centrifuge and 400 μ L of clarified supernatant was transferred to a fresh Eppendorf tube.



Figure 17. *Left*: Sample sites on Long Island. *Right*: Concentration of whole water (3L capacity) via PVC device.

Field qPCR: The appropriate number of Phyttoxigene™ DinoDTec tubes with lyophilized qPCR reagents were each re-suspended with 80 µL molecular biology grade (MBG) water. After vortexing for ~10 sec, aerosol-barrier pipet tips were used to combine all re-suspended reagents into one tube: the composite was briefly vortexed and centrifuged. 20 µL were aliquoted into individual reaction tubes and 5 µL of template (stock or diluted supernatant in MBG water) or MBG water (negative control) or at least one standard supplied with the DinoDTec kit (positive control) were added. The reaction tubes were briefly centrifuged and run on a MyGo Mini qPCR machine. Cycling was per manufacturer’s directions as described above. For the Weesuck Creek day 3 sample, a high silt load impacted the two pre-filtration steps (150 and 11 micron), so the units had to be rinsed vigorously in a bucket of water throughout the process. A generator failure on the last sampling day resulted in qPCR samples being stored on wet ice and run back in the laboratory at the end of the day.

Table 5. Cell counts and supporting environmental metadata for samples collected from three sites over a three day period in Long Island Sound, NY.

Date	Sample ID	Location	Alexandrium Cells/L	Temperature	Salinity	DO %
5-7-19	LI 01	Meetinghouse Creek	2,900	16.5	24.7	51.5
	LI 02	James Creek	7,600	15.5	25.5	50.5
	LI 03	Weesuck Creek	4,800	14	30.1	58.0
5-8-19	LI 04 (field blank)	James Creek	nd	18.5	24.1	68.1
	LI 05	James Creek	nd	18.5	24.1	68.1
	LI 06	Meetinghouse Creek	150	21.2	4.03	31.1
	LI 07 (field duplicate)	Meetinghouse Creek	550	21.2	4.03	31.1
	LI 08	Weesuck Creek	12,700	19.6	29.2	150.3
5-9-19	LI 09	James Creek	50,500	18.1	23.7	86.3
	LI 10	Meetinghouse Creek	1,350	19.4	13.9	116.5
	LI 11	Weesuck Creek	26,650	19.6	28.7	139.9

There was some variability for sxtA detection between the two platforms (Figure 18) which could be related to the sampling environment, or sample processing complications on a few occasions. For example, positive qPCR on both platforms with no *Alexandrium* cell counts for sample LI05 may reflect the detection of a different saxitoxin-producing dinoflagellate(s). The discrepancy for sample LI09 may be explained by a generator failure: this was the first sample of the day and the lysate had to be stored on wet ice until the end of the day when qPCR was carried out back at the lab on the MyGo Mini. BDL for LI06 and LI07 on both platforms reflect the low number of cells in the reaction: 5 cells and 25 cells for LI06 on the MyGo Mini and Quanta Bio Q,

respectively; 20 cells and 92 cells for LI07 on the MyGo Mini and Quanta Bio Q, respectively. It is unclear why the reference sample (Quanta Bio Q) was BDL for LI10.

Saxitoxin target gene (*sxtA*) results from both platforms is plotted against ELISA results in Figure 18 and correlates well through sample LI07. An offset in values for the remainder of the samples may be explained by the physiological state of the cells; cells may have not been producing much toxin (samples LI08 and LI10), or perhaps fewer cells were producing increased toxin (samples LI09 and LI11; however note that qPCR signal may have been compromised for LI09-LI11 by a delay in running the lysates due to the generator failure). Data are also presented as cross-plots of cell counts (Figure 19; *Alexandrium*) and toxin analysis (Figure 20) against *sxtA* qPCR on both platforms.

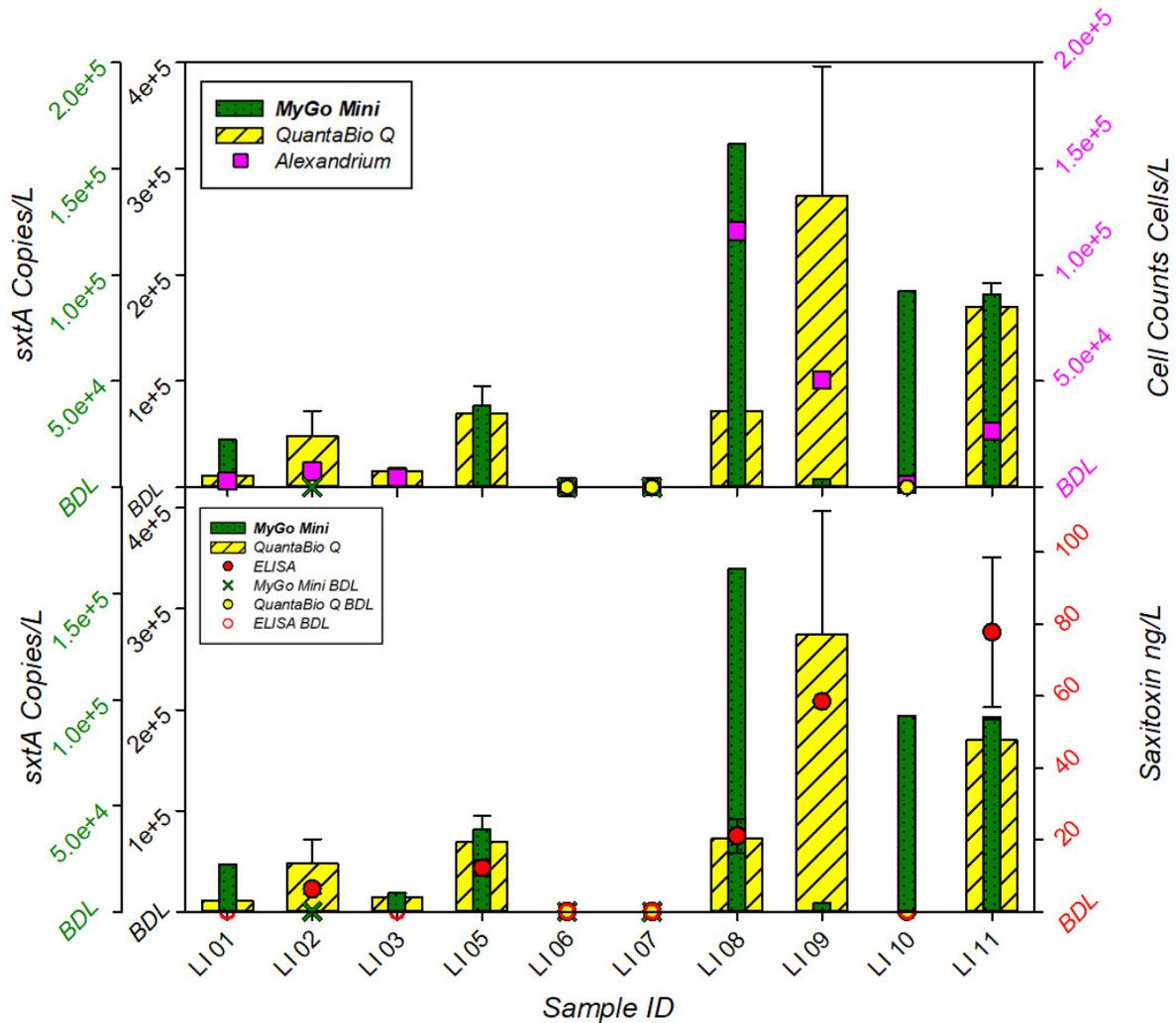


Figure 18. *Top Panel:* *sxtA* qPCR detection from the MyGo Mini (green bars, outer left axis), QuantaBio Q (yellow bars, inner left axis), and *Alexandrium* counts (pink squares, right axis) for Long Island field samples outlined in Table 5. No sample was collected for cell counts for LI 05. *Bottom Panel:* *sxtA* qPCR detection from the MyGo Mini (green bars, outer left axis), QuantaBio Q (yellow bars, inner left axis), and ELISA saxitoxin estimates (red circles, right axis.) BDL = Below Detection Limit. qPCR for LI04 (field blank) was negative on both platforms (data not shown). Error bars represent one standard deviation of the average of two analytical reps from each of two filter replicates (n=4) for the reference samples (QuantaBio Q).

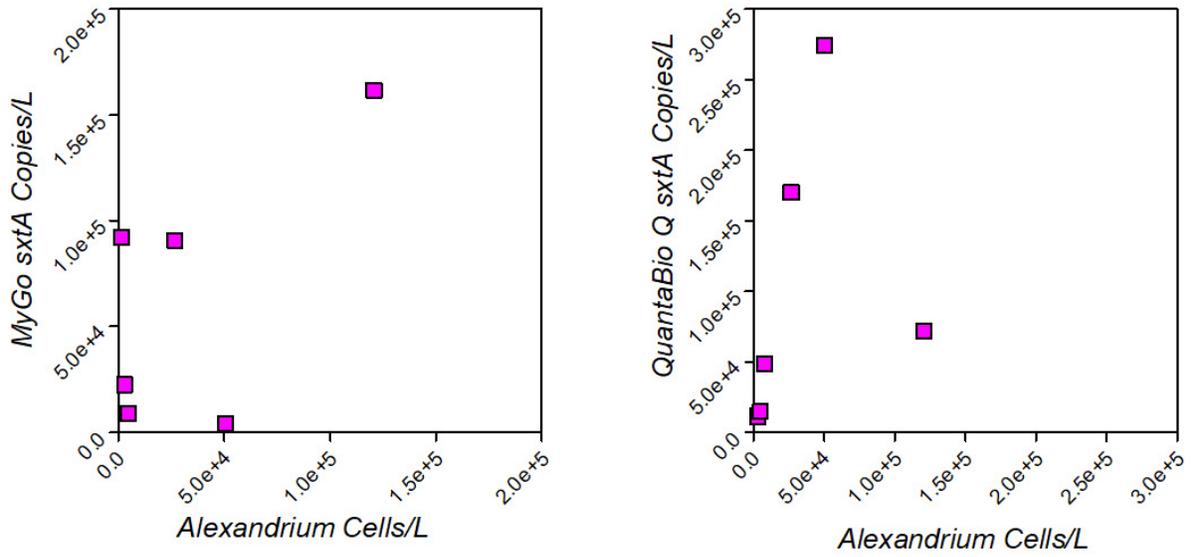


Figure 19. Cross plots of MyGo Mini and QuantaBio Q (reference) estimates of sxtA copies/L and *Alexandrium* cell counts.

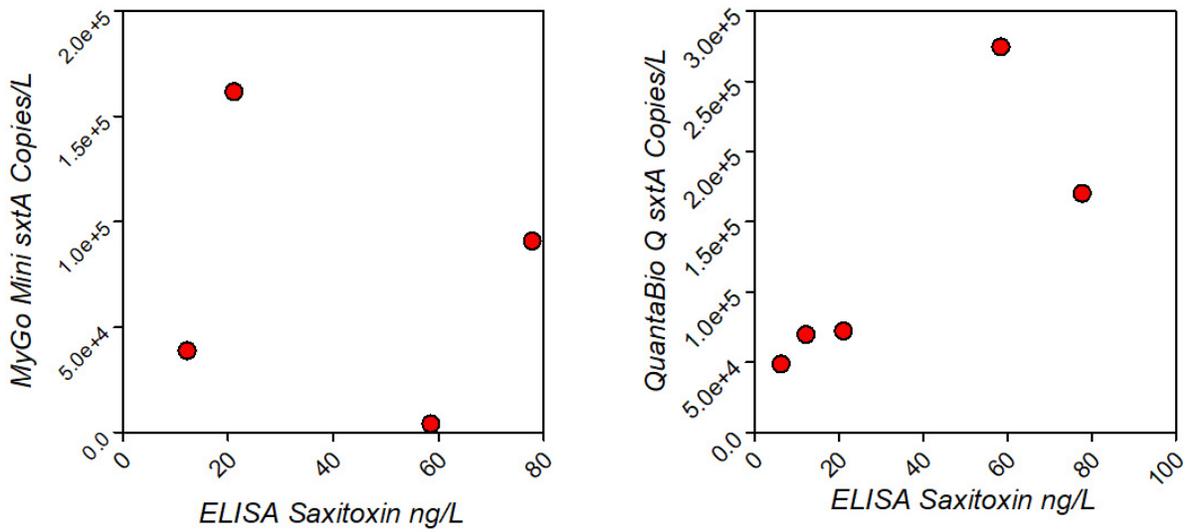


Figure 20. Cross plots of MyGo Mini and QuantaBio Q (reference) estimates of sxtA copies/L and ELISA for saxitoxin.

Western Lake Erie and Sandusky Bay, Great Lakes

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 21). 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 21). 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.

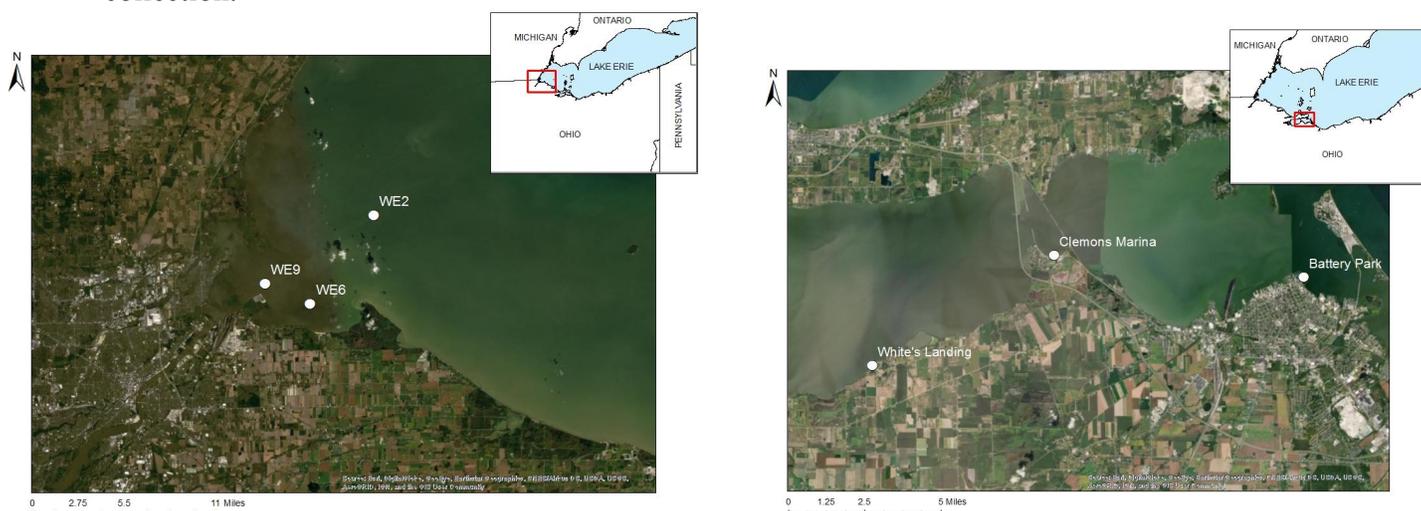


Figure 21. Western Lake Erie and Sandusky Bay sample stations. Western Lake Erie sampling stations (left) for WE2, WE6, and WE8. Sandusky Bay sampling locations (right) for Whites Landing, Clemons Marina and Battery Park.

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 6). Reference sample qPCR results for 16S and *mcyE/ndaF* gene markers are presented in Figure 22. It is interesting to note that despite the significantly higher amount of toxin, 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* with the in-field tested samples on the MyGo Pro platform (Figure 23). We again observed the order of magnitude difference between qPCR signal from the MyGo Pro platform versus the QuantaBio Q reference standards (note scale differences on primary y-axis in Figure 23) which likely experienced some loss of genetic material due to the multi-step, column-based full DNA extraction protocol. Data are also presented as cross-

plots of cell counts (Figure 24; *Microcystis* + *Planktothrix*) and toxin analyses (Figure 25) against *mcyE/ndaF* qPCR on both platforms.

Table 6. 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 1.5 µg/L of a dissolved MC standard. Sample GL16 was made by spiking GL13 with 1.5 µg/L of a dissolved MC standard (Abraxis Microcystins/Nodularins (ADDA) spiking solution, Abraxis IC 300702). Spiking of microcystin standards was performed as part of testing an un-related platform and had no bearing on toxin gene concentration.

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 – the day prior to sampling because chlorophyll samples were not processed from the day of collection.

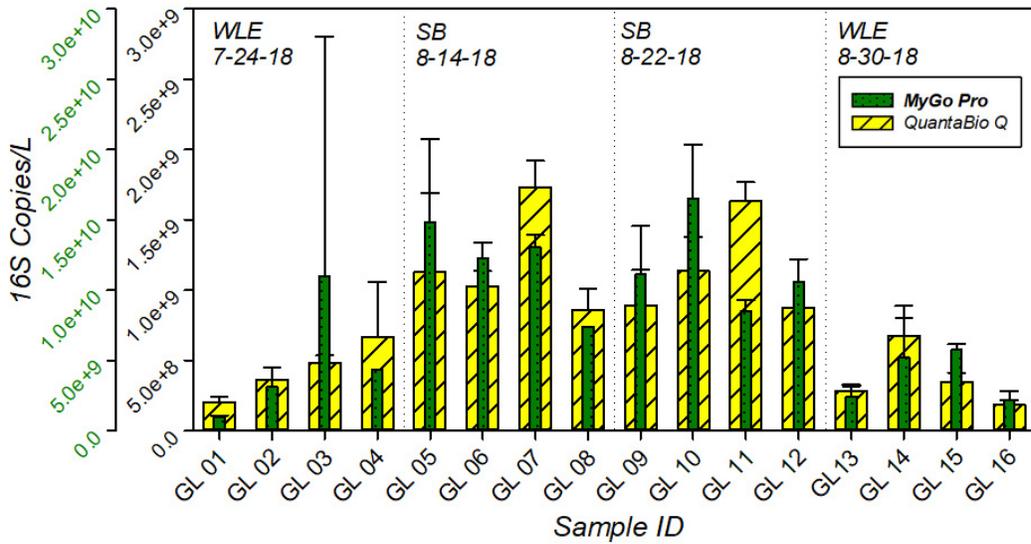


Figure 22. Estimates of cell density in gene copies per liter derived from the MyGo Pro (green bars, far left axis) and QuantaBio Q (yellow bars, near left axis). Results are given for 16S total cyanobacteria copies per liter. Error bars represent one standard deviation of the average for the two sample replicates and the two analytical replicates of a given sample extract (n = 4).

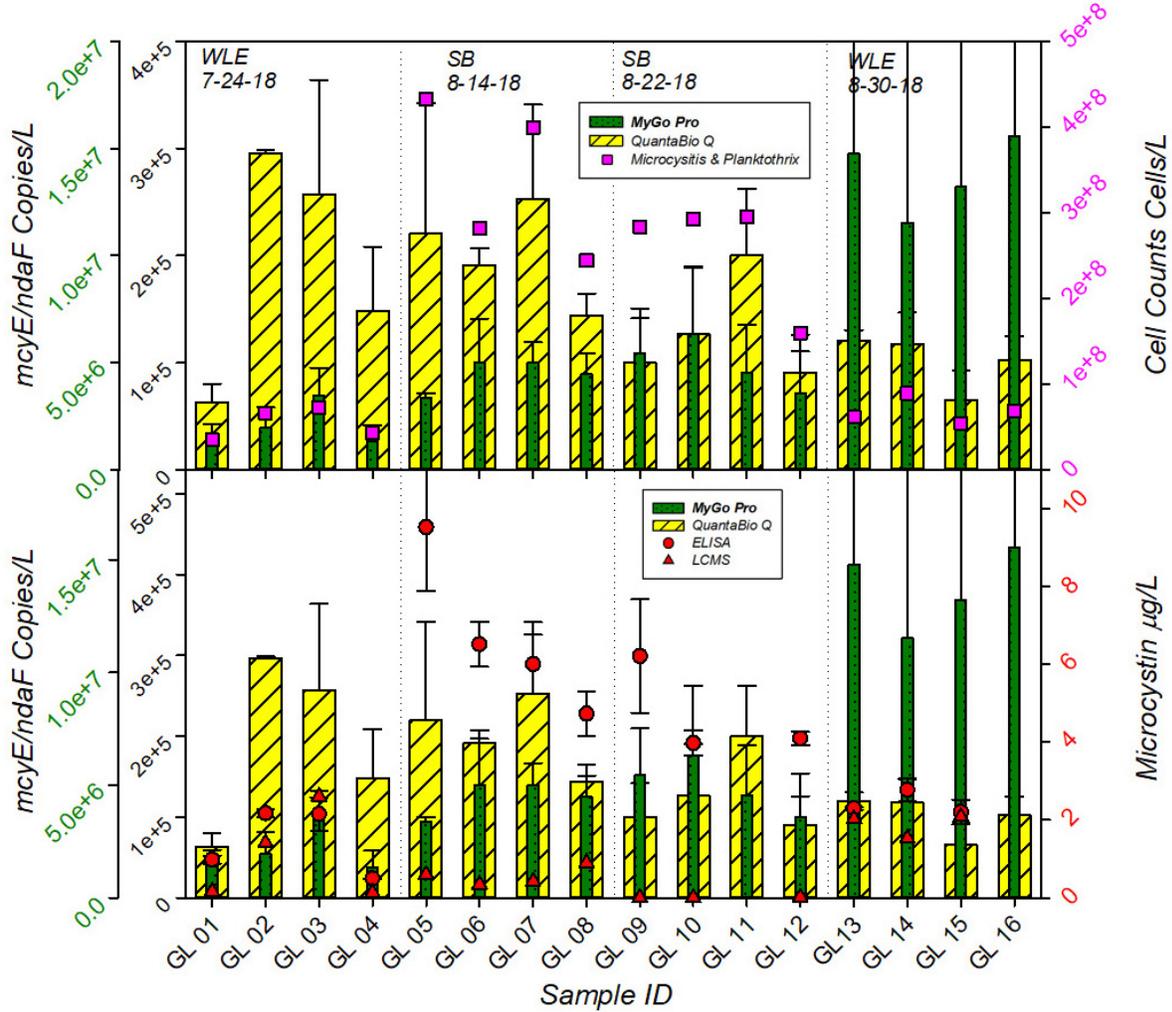


Figure 23. qPCR gene copies as measured on both the MyGo Pro (green bars, far left axis) and QuantaBio Q (yellow bars, near left axis) for the Great Lakes field deployment outlined in Table 6. *Top Panel:* Copies of *mcyE/ndaF* (toxin genes) compared to *Microcystis* and *Planktothrix* counts in cells/L (pink squares, right axis.) *Bottom Panel:* Copies of microcystin/nodularin toxin production genes (*mcyE/ndaF*) plotted against microcystin estimates from ELISA (red circles) and LCMS (red triangles). Error bars represent one standard deviation of the average of two analytical reps from each of two filter replicates (n=4). Toxin data for GL 11 and GL 16 are not included as those samples were spiked with microcystin standard.

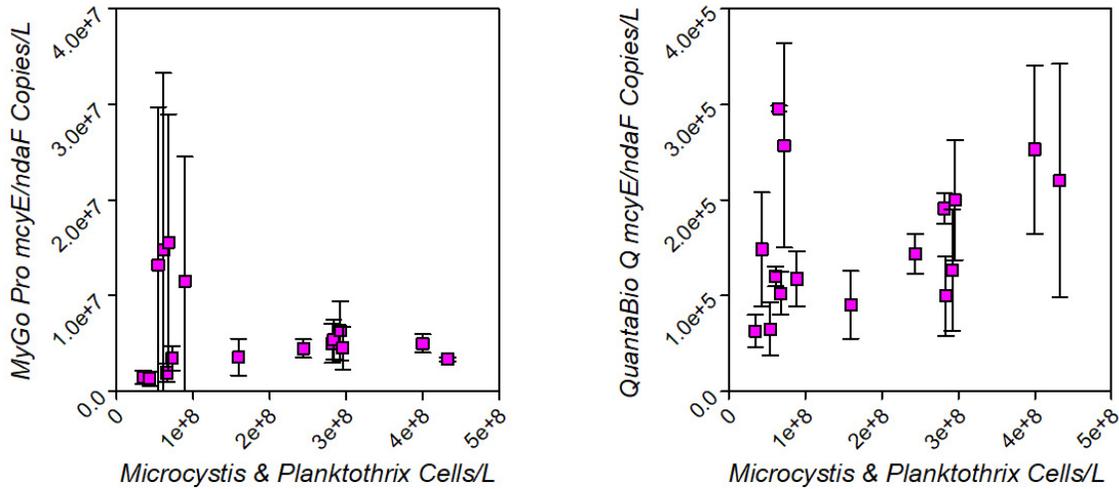


Figure 24. Cross plots of MyGo Pro (left) and QuantaBio Q (right) qPCR estimates of *mcyE/ndaF* copies/L and cell counts for *Microcystis* and *Planktothrix*.

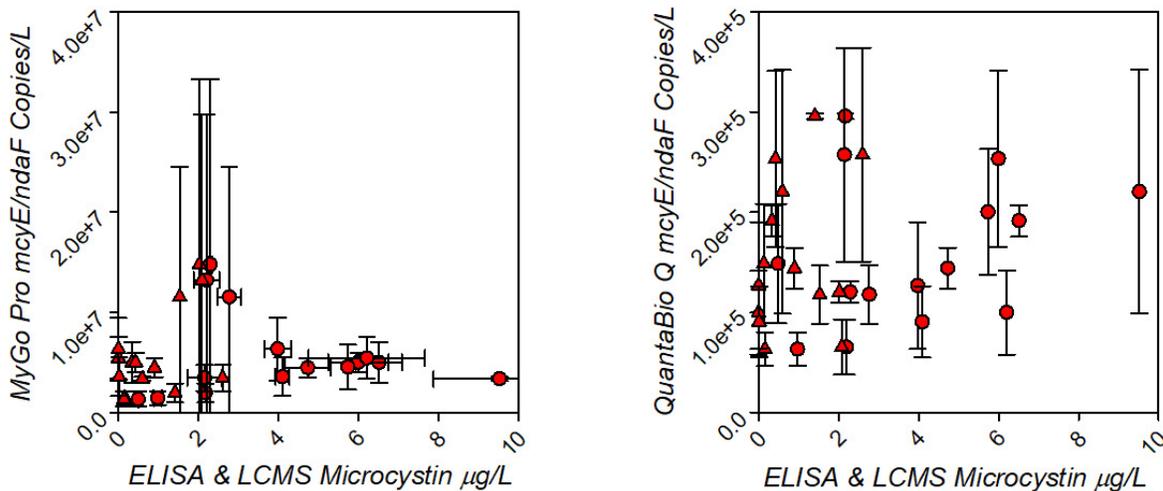


Figure 25. Cross plots of MyGo Pro (left) and QuantaBio Q (right) qPCR estimates of *mcyE/ndaF* copies/L and ELISA (circles) and LCMS (triangles) microcystin estimates.

Monterey Municipal Wharf, California

During the testing period, concentrations of saxitoxin-producing 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 target depths and were then 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 seven samples generated (Table 7), including one field duplicate and one matrix spike with cultured *Alexandrium* cells (30 cells/mL final concentration). In order to concentrate low densities of naturally occurring *Alexandrium* cells, samples (indicated in Table 7)

were first concentrated into smaller volumes via filtration of a fixed volume through the PVC unit (Figure 17, right panel), followed by sterile filtered seawater (FSW) flushing of material off the screen into a clean container with addition of FSW to a desired volume for processing. For samples ML-21A and ML-21B, 8L of water were first concentrated and the material flushed from the filter screen was brought up to a final volume of 500mL. For samples ML-25 and ML-26, 3L of water were first concentrated and the material flushed from the filter screen was brought up to a final volume of 1L.

Table 7. Field samples collected from the Monterey Municipal Wharf in fall 2018.

Date	Sample ID	Description	Volume filtered (mL)	Alexandrium Cell/L	Chlorophyll µg/L
9-19-18	ML - 20	un-concentrated	100	6,000	-
9-20-18	ML - 21A	Concentrated	50	12,000	2.47
9-20-18	ML - 21B (field dup)	Concentrated	50	12,000	2.47
9-26-18	ML - 25	Concentrated	140	27,000	2.70
9-26-18	ML - 26	Concentrated	140	25,000	2.70
10-4-18	ML - 31	un-concentrated	2000	12,000	7.70
10-4-18	ML - 32	Spiked	2000	20,000	6.06
10-4-18	ML - 32D	1:10 Dilution	n/a	n/a	n/a

The volumes used for concentrating naturally occurring saxitoxin-producing dinoflagellate cells from the Monterey Municipal Wharf sampling were not adequate for detection by qPCR on either platform (Figure 26). In order to increase the volumes filtered for qPCR (Table 7), tens of liters would have needed to be concentrated, far beyond the 2-3L used in most field sampling protocols. In the samples where cells were detectable but saxitoxin and sxtA gene targets were negligible or BDL, it could be that non-toxic *Alexandrium* spp. (or morphologically similar species) were present. Conversely, where cells and saxitoxin were detectable but qPCR gene targets were negligible it could be that the number of targets present fell below detection level for the assay. In particular, there may have been loss of material during the concentration steps.

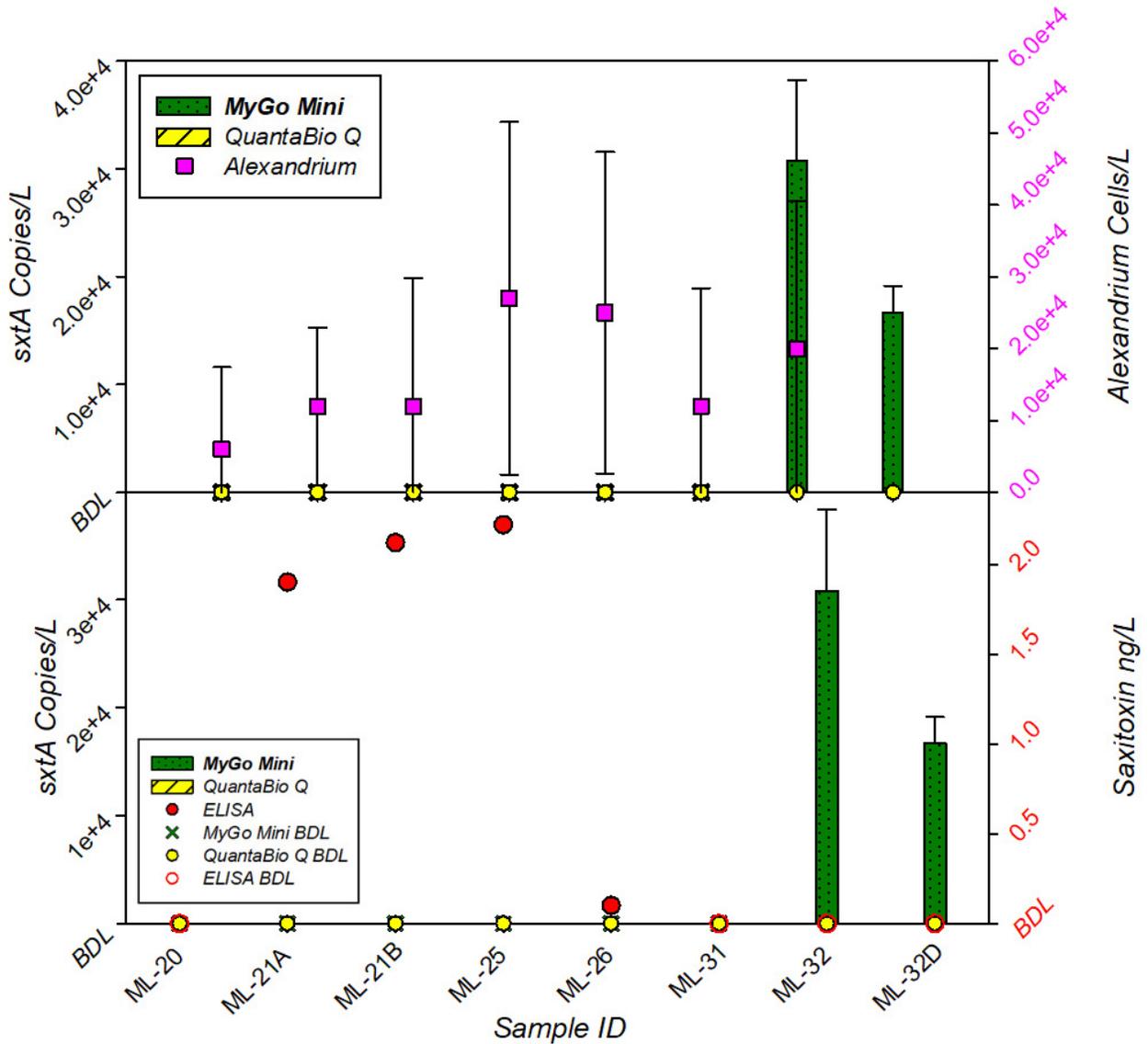


Figure 26. qPCR gene copies as measured on both the MyGo Mini (field samples, green bars) and QuantaBio Q (reference samples, yellow bars) for the Monterey Municipal Wharf field deployment outlined in Table 7. *Top Panel:* sxtA copies/L estimated by MyGo Pro and QuantaBio Q compared to Alexandrium counts in cells/L (pink squares, right axis). *Bottom Panel:* sxtA copies/L estimated by MyGo Pro and QuantaBio Q compared to ELISA saxitoxin estimates (red circles, right axis).

Pinto Lake/Freshwater systems, California

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 ML-14 on September 17th; samples ML-16 through ML-19 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 ML-15 on September 17th). Samples ML-18 and ML-19 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.

qPCR analysis indicated that the total cyanobacterial densities, (estimated by copies of the 16S gene marker; Figure 27) were 1-2 orders of magnitude lower than seen in the Lake Erie field trials. Note scale differences between the two qPCR platforms in Figure 27. Copies of *mcyE/ndaF* gene markers were all below quantification (data not shown). Small levels of microcystin were detected occasionally by LCMS-LR (1.3-2.5 $\mu\text{g/L}$) but not by ELISA (data not shown).

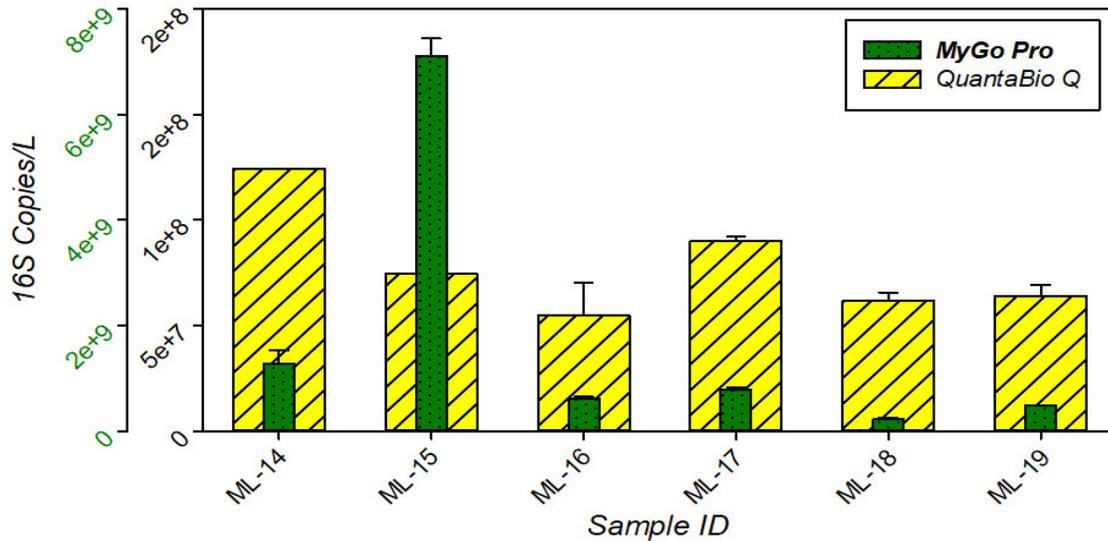


Figure 27. qPCR results for Pinto Lake and El Estero Lake (ML-15) for 16S on both platforms. Copies of the *mcyE/ndaF* gene markers were all below quantification (< 45 copies per reaction) so no data are presented. Error bars are one standard deviation of two filters and two analytical replicates (n = 4).

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 19, 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 8. 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 8. 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 8). The targeted spike by known addition was 0.5 µg/L of dissolved Microcystins/Nodularins (ADDA) 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 8. Results of independent field duplicates and spike recoveries for 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 evaluation. The objectives of the TSAs conducted during this evaluation 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 indicating 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 was 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 evaluation 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 9. 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 9. 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									Cell Counts ^{4/}
			ELISA ^{3/}			LCMS-LR			qPCR ^{3/}			
			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 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 evaluation'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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January 5, 2021

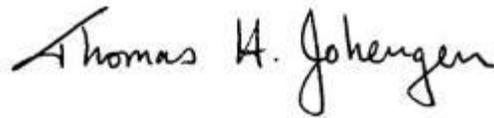
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Approved By: Dr. Mario Tamburri
ACT Executive Director

January 5, 2021

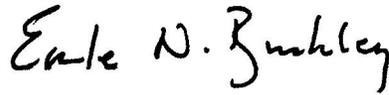
Date



Approved By: Dr. Tom Johengen
ACT Chief Scientist

January 5, 2021

Date



Approved By: Dr. Earle Buckley
Quality Assurance Supervisor



Thursday, January 14, 2021

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**Alliance for Coastal Technologies Toxin Verification Report
Phytoxigene™ DinoDTec and CyanoDTec quantitative real-time PCR (qPCR) kits**

Manufacturer's Response: Phytoxigene, Inc.

Phytoxigene appreciated the opportunity and engagement by the Alliance of Coastal Technologies in assessing its CyanoDTec and DinoDTec kits. While both kits have been commercially available for a number of years, they had not yet been verified for their utility and compatibility for field use. In the original design of the two assays, considerable thought was given to ensuring the kits were formatted in a way that allowed for a simple and easy methodology that could potentially be used by both, experienced laboratory personal and non-experts, for rapid field analysis. The HAB community have consistently maintained a need to have such tools be available to them. The demonstration process undertaken by ACT, and subsequent verification report is therefore both welcomed and appreciated.

Our internal data along with other external evaluations is consistent with a number of key aspects of the report, specifically the use of a lysate, rather than a complete nucleic acid extraction protocol, yields higher levels of genomic material and as gene copy number increases, toxin levels generally increase comparably. The report highlights the need to consider the relationship between gene copy number and toxin levels as inductive and highly dependent on sample processing protocols. Logically, standardization of these protocols are a significant consideration when adopting qPCR methods for ongoing monitoring programs

While the two PCR platforms and extraction protocols showed consistent differences, it should be noted that the copy number consistently varied between 0.5-1 log, in cq value terms, this is a difference of 1-3 cycles. In many testing environments, this difference is not significant, especially when detecting high levels of toxin genes.

We believe the report highlights that the demonstration has successfully met its objectives, and are pleased by the overall summary that the kits are easily adapted to various field applications for both expert and non-expert individuals.

I would take this opportunity to express our appreciation to the whole team for their dedication and time working on this program. We found the collaboration extremely worthwhile, and would recommend it to other companies with emerging technologies wanting to gain knowledge and experience in how their kits would work in real world situations.

Yours sincerely

A handwritten signature in black ink, appearing to read "M. Van Asten". The signature is fluid and cursive, written over a large, faint, light-colored watermark of a stylized leaf or flower shape.

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