QPCR A SCREENING TOOL FOR CYANOBACTERIA

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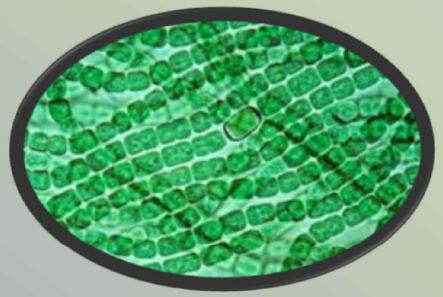
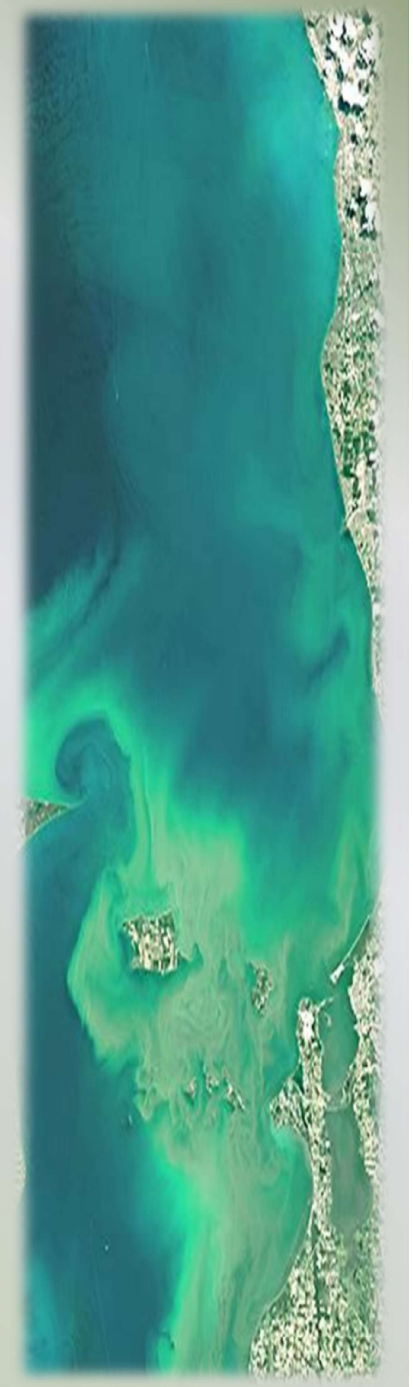
Northeast Ohio Regional Sewer District

Agenda

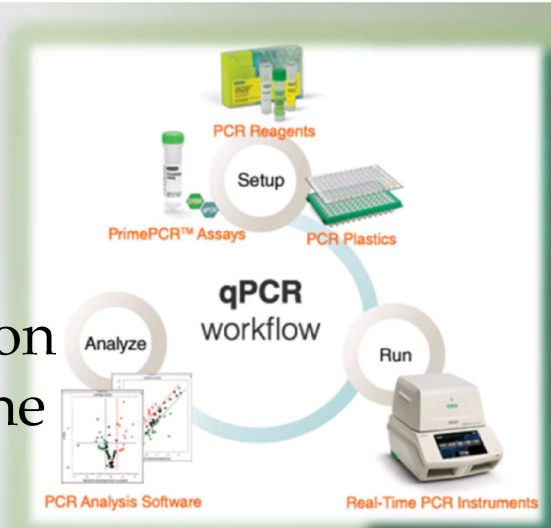
- qPCR and Environmental Monitoring
- Benefits of qPCR Technology
- NEORSD Cyanbacteria Workflow
- CyanDtec Assay
- Data Generated using the CyanDtec Assay

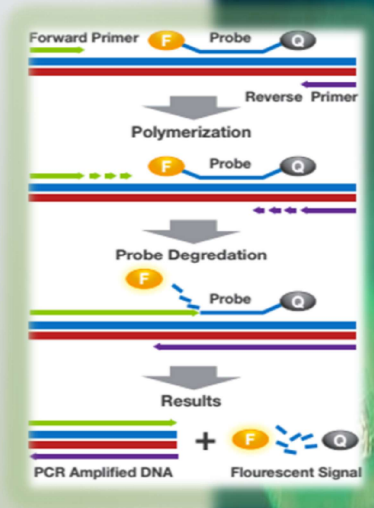


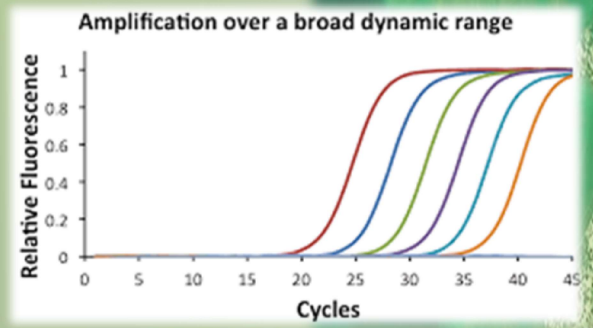


What is qPCR

- Quantitative Polymerase Chain Reaction (qPCR) is a technique used to determine the exact concentration of number of DNA sequences amplified
  - Quantitation can be Relative or Absolute
- qPCR is sometimes called "molecular photocopying" because the technique copies (amplifies) a small segment of DNA from a target sequence exponentially
- qPCR is avery powerful and can reduce the time needed to identify pathogenic organism

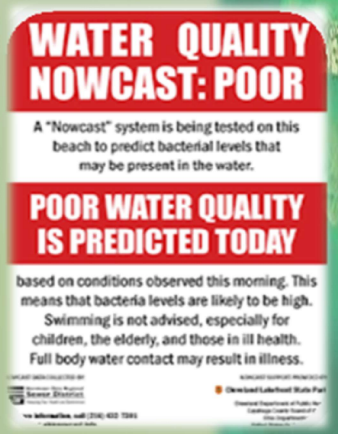






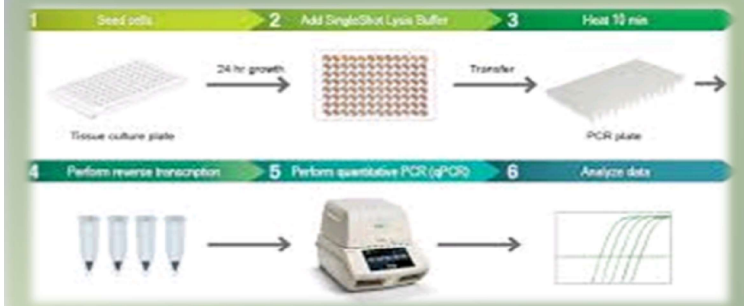
Water Quality Monitoring

- ✓ EPA has published methods using qPCR technology for recreational WQ monitoring for Fecal Indicator Bacteria (*E. coli*, Enterococci)
  - ✓ BEACH Act 2000: Beaches Assessment and Coastal Health Act (BEACH Act) required the EPA to provide or develop "appropriate, accurate, and expeditious, cost-effective for WQ monitoring
  - ✓ 2003 NEEAR Study National Epidemiological and Environmental Assessment of Recreational Study compared results from traditional culture methods and rapid genetic (qPCR)
  - ✓ 2010 SCCWRP: Southern California Coastal Water Research Project started a 3 year project comparing qPCR and culture based assays
- ✓ 2012 Recreational Water Quality Criteria included the use of qPCR methods and WQ limits based on qPCR
  - ✓ EPA Methods 1609 and EPA 1611 for Enterococci

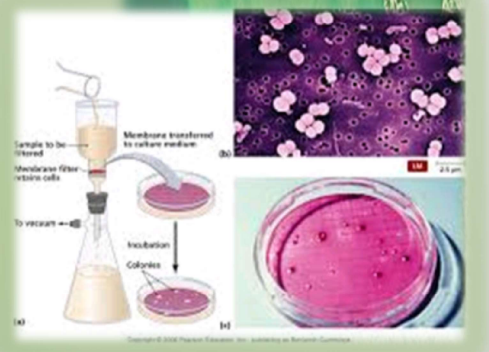


qPCR vs Culture Methods

- ✓ qPCR
  - ✓ Results in 3 – 4 hours
  - ✓ Single extract can be used for multiple FIB (*E. coli* and Enterococci)
  - ✓ 6 hour hold time for filtration
  - ✓ Extracts can be frozen for later analysis
  - ✓ Instrument can analyzed multiple samples simultaneously
  - ✓ Measures both living and non-living cells
  - ✓ Initial cost of instrumentation and supplies is expensive
  - ✓ Expertise is needed

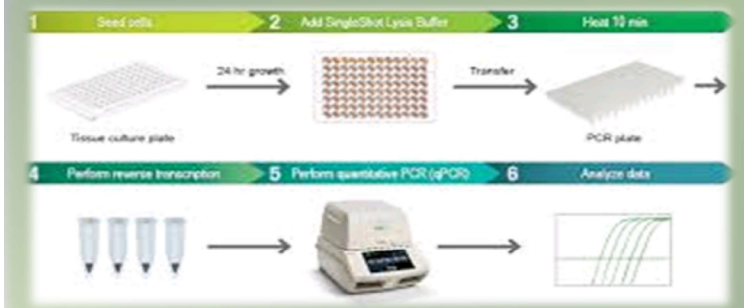


- ✓ Culture Methods
  - ✓ Results in 18 to 24 hours
  - ✓ Different reagents and media needed for each FIB (*E. coli* and Enterococci)
  - ✓ 6 hour hold time for analysis
  - ✓ Samples are consumed after analysis
  - ✓ Samples analyzed manually
  - ✓ Measures living cells or cells that can multiply
  - ✓ Upfront cost lower however more supplies and reagents needed
  - ✓ General knowledge of microbiology
  - ✓ **Accepted Methods**

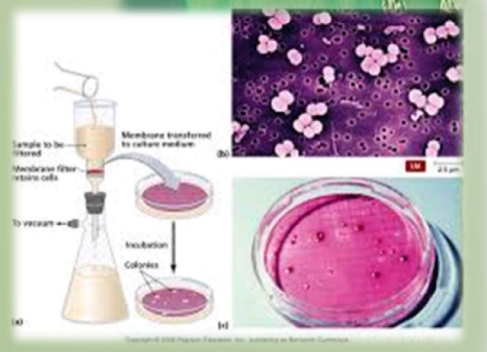


qPCR vs Algae Identification

- ✓ qPCR
  - ✓ Confirms Cyanobacteria presence by DNA (16SrRNA)
  - ✓ Does not identify species
  - ✓ Number of gene copies not equal to the number of cells
  - ✓ Determines if the toxin producing gene is present
  - ✓ Sample preparation is simple and quick
  - ✓ Expertise is needed



- ✓ Algae ID /Enumeration
  - ✓ Confirms Cyanobacteria by morphology
  - ✓ Identify species
  - ✓ Sample can be enumerated
  - ✓ Cannot determine if the species can produce toxins
  - ✓ Multiple toxins can be produced form a single species
  - ✓ Concentration of sample can take 2 – 6 hours
  - ✓ Some expertise is needed
  - ✓ **Accepted Method**

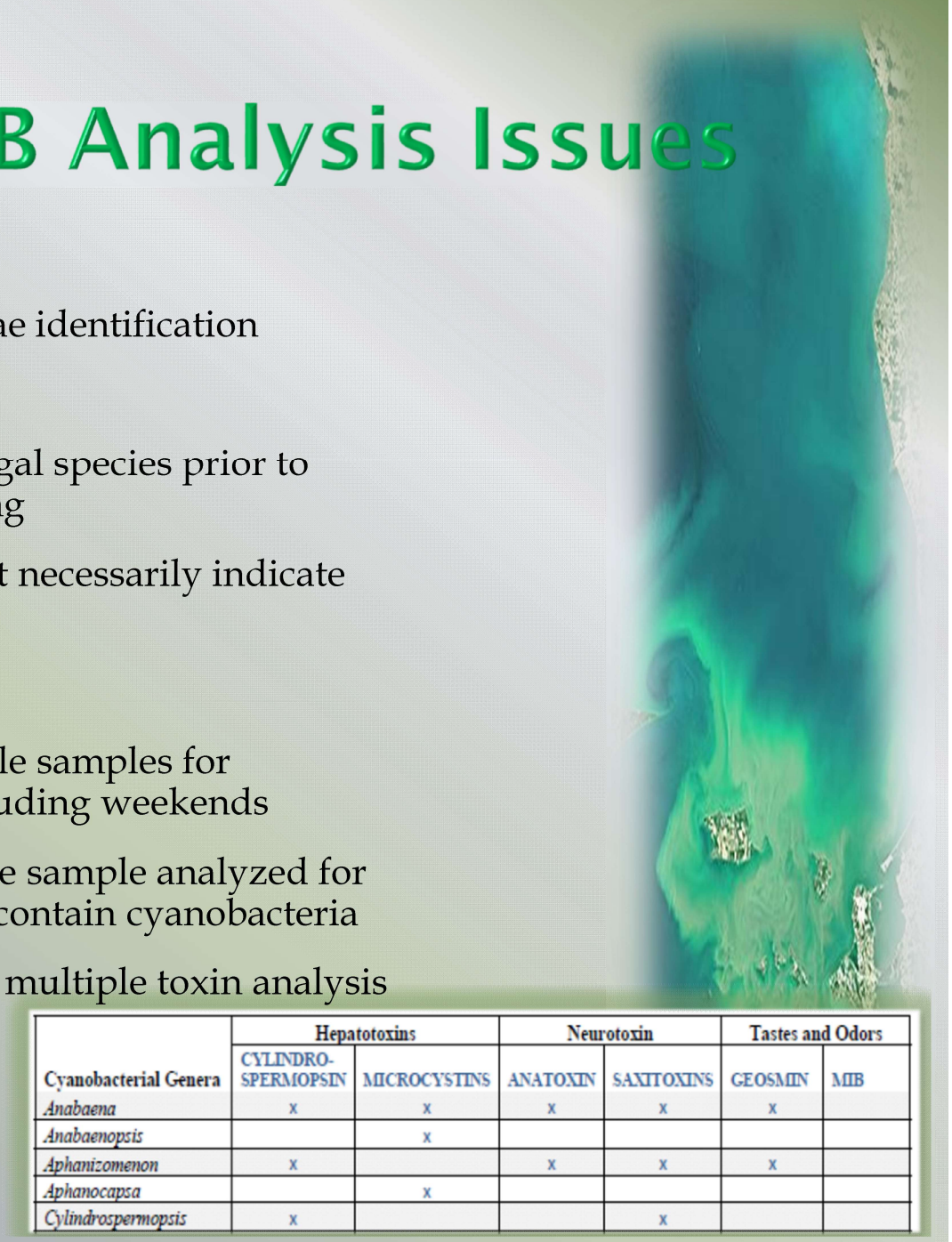


Methods for Toxin Analysis

- ✓ Most laboratories use the ELISA method to quantify the following cyanotoxins; cylyndrospermopsin, microcystins, anatoxin, and saxitoxin
  - ✓ Ohio EPA Total (Extracellular and Intracellular) Microcystins - ADDA by ELISA Analytical Methodology Version 2.0, January 2015
  - ✓ Analysis cost is approximately $50 - $125 per analysis per toxin
- ✓ Some laboratories use liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) to quantitate the various toxins and differentiate between the various congeners.
  - ✓ EPA Method 544 Determination of Microcystins and Nodularins in Drinking Water by Solid Phase Extraction and Liquid Chromatography / Tandem Mass Spectrometry (LC/MS/MS)
  - ✓ EPA Method 545 Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS)
  - ✓ Analysis cost can range from $300 – $500 per sample per method

2014/2015 HAB Analysis Issues

- ✓ Staffing
  - ✓ Three analysts skilled with algae identification
  - ✓ Single microscope
  - ✓ Identifying the predominate algal species prior to toxin analysis is time consuming
  - ✓ Identifying the species does not necessarily indicate the toxin
- ✓ Work load
  - ✓ Increasing sample load, multiple samples for identification and analysis including weekends
  - ✓ Clients did not want to have the sample analyzed for the toxin if the sample did not contain cyanobacteria
  - ✓ Clients did not want to pay for multiple toxin analysis
  - ✓ Developing a backlog



Cyanobacterial Genera	Hepatotoxins		Neurotoxin		Tastes and Odors	
	CYLINDRO-SPERMOPSIN	MICROCYSTINS	ANATOXIN	SAXITOXINS	GEOSMIN	MIB
Anabaena	x	x	x	x	x	
Anabaenopsis		x				
Aphanizomenon	x		x	x	x	
Aphanocapsa		x				
Cylindrospermopsis	x			x		

Problem Statement

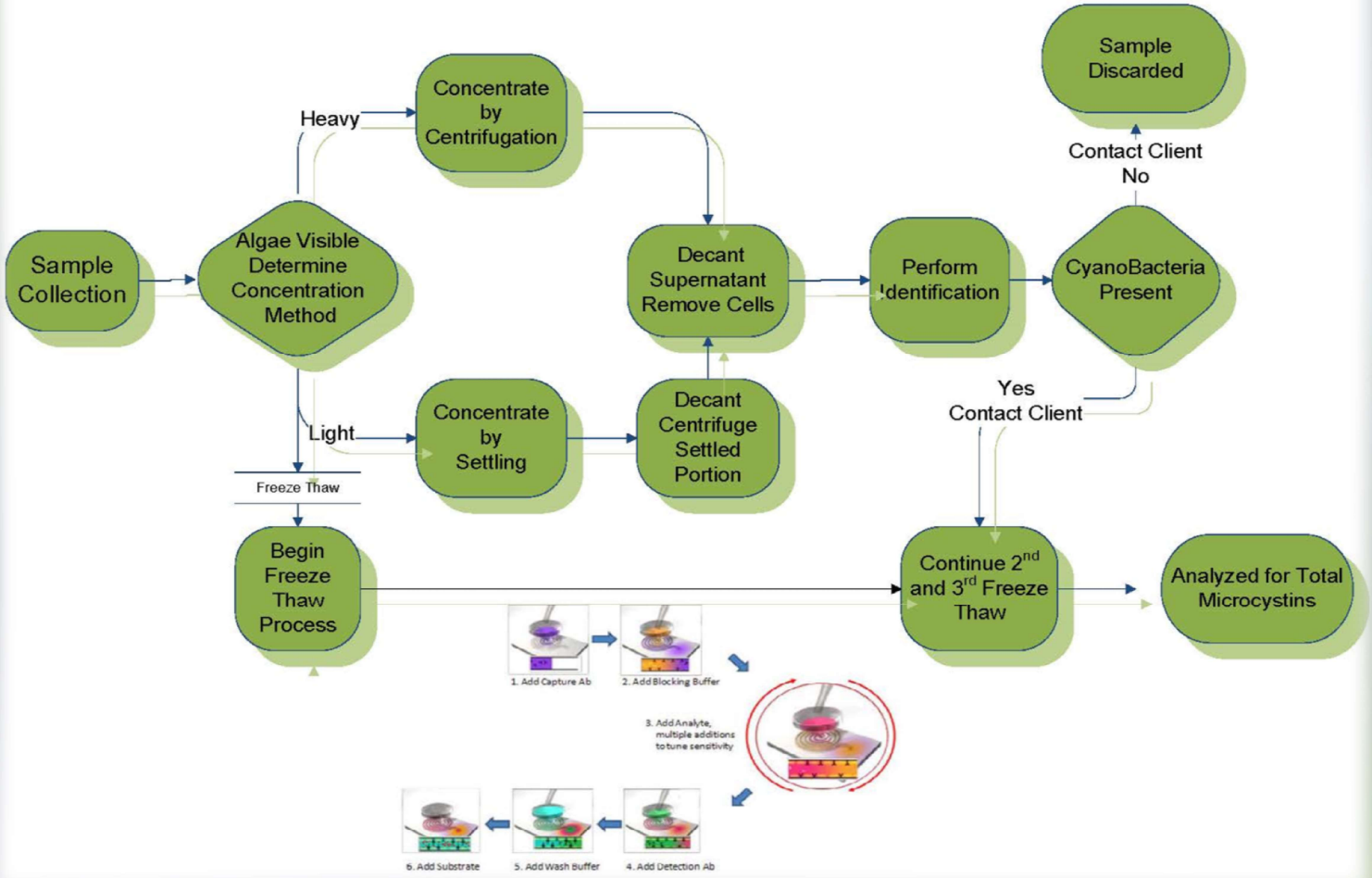
- ✓ Cyanobacteria are capable of producing multiple toxins or none at all
- ✓ Identification alone does not indicate what toxin if any the organism may produce
- ✓ Identification requires skill and can be time consuming
  - ✓ Samples may need to be concentrated for accurate identification adding analysis time

Cyanobacterial Genera	Hepatotoxins		Neurotoxin		Tastes and Odors	
	CYLINDRO-SPERMIOPSIN	MICROCYSTINS	ANATOXIN	SAXITOXINS	GEOSMIN	MIB
Anabaena	×	×	×	×	×	
Anabaenopsis		×				
Aphanizomenon	×		×	×	×	
Aphanocapsa		×				
Cylindrospermopsis	×			×		
Fischerella					×	
Haplosiphon		×				
Hyella					×	×
Lyngbya (Plectonema)	×			×	×	×
Microcystis		×				
Nostoc		×			×	×
Oscillatoria (Planktothrix)		×	×	×	×	×
Phormidium			×		×	×
Pseudanabaena		×				×
Raphidiopsis	×		×			
Schizothrix						
Umezakia	×					
Synechococcus		×			×	×
Synechocystis		×				

Information adapted from Jennifer Graham, USGS

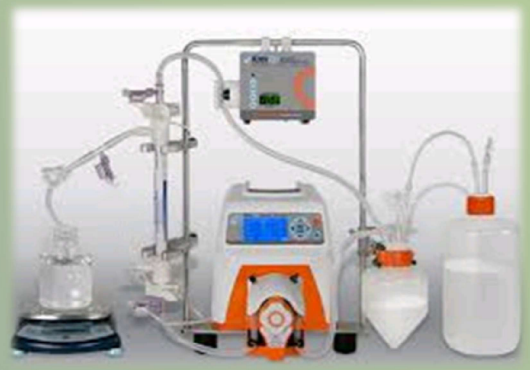
Cyanotoxin Workflow

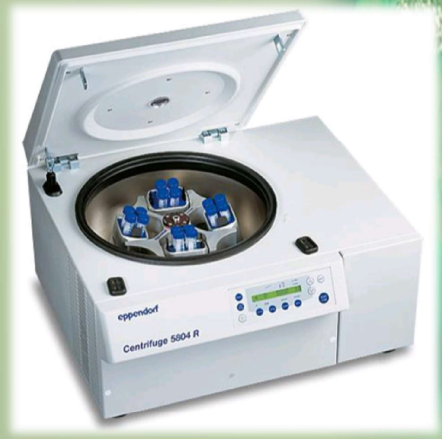
Cyanobacteria Identification Workflow



Workflow Improvement

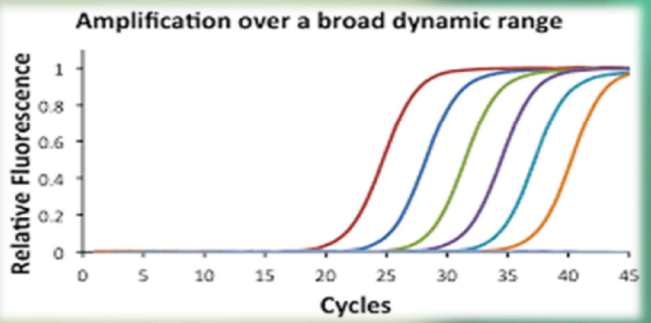
- ✓ Identification and enumeration
- ✓ Rapid concentration method for the
  - ✓ Membrane filtration
  - ✓ Tangential flow filtration (TFF)
  - ✓ Utermohl Chambers
  - ✓ Centrifugation
- ✓ Use of molecular methods
  - ✓ qPCR





qPCR Research

- ✓ USGS
  - ✓ Relations Between DNA- and RNA-Based Molecular Methods for Cyanobacteria and Microcystin Concentration at Maumee Bay State Park Lakeside Beach, Oregon, Ohio, 2012
- ✓ Water Research Foundation
  - ✓ Early Detection of Cyanobacterial Toxins Using Genetic Methods [Project #2881]
- ✓ 2015 US Algal Conference (Akron, Ohio)
  - ✓ Phytoxigene™; Molecular detection and quantification of biotoxin producing Genes



Phytoxigene™

- ✓ CyanoDTec Assay
  - ✓ Multiplexed qPCR assay that quantifies the genes for total cyanobacteria and three specific toxin genes
    - ✓ Total Cyanobacteria; (16S rRNA) with an Internal Amplification Control (IAC)
    - ✓ Toxin Gene Kit; microcystin/nodularin, cylindrospermospin, and saxitoxin
- ✓ CyanoNAS Standard Kit
  - ✓ 6 Standards 100ul each

Standard	Copies/ul	Copies per reaction
NAO11	0 copies/ul	0 copies (BLANK)
NAO12	20 copies/ul	100 copies/reaction
NAO13	200 copies/ul	1,000 copies/reaction
NAO14	2,000 copies/ul	10,000 copies/reaction
NAO15	20,000 copies/ul	100,000 copies/reaction
NA026	200,000 copies/ul	1,000,000 copies/reaction

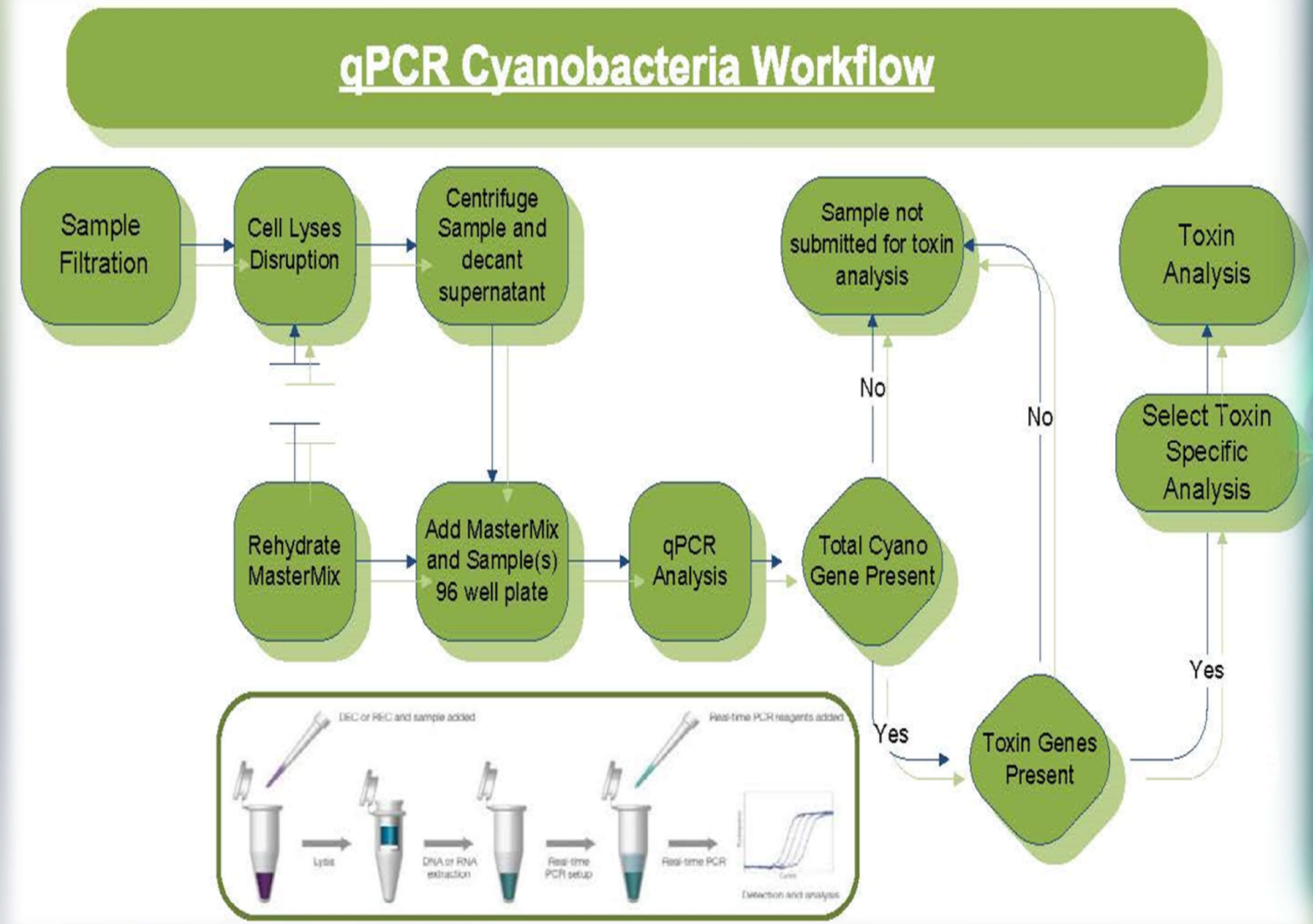
Product #	Description and Target	Contents	# Tests
205-0050	**Phytoxigene™ CyanoDTec Total Cyanobacteria**; (16S rRNA) and Internal Amplification Control (IAC) target	6 tubes each containing all necessary reagents (enzymes, probe, primers and dNTP) for 4 tests	24
205-0051	**Phytoxigene™ CyanoDTec Toxin Gene**; microcystin/nodularin, cylindrospermospin and saxitoxin	6 tubes each containing all necessary reagents (enzymes, probe, primers and dNTP) for 4 tests	24

Phytoxigene™

- ✓ Platform
  - ✓ Applied Biosystems (ABI) 7500
  - ✓ ABI StepOne Plus
  - ✓ Cepheid SmartCycler
- ✓ Cycle Parameters
  - ✓ Initial denaturation 95 °C, 2 minutes
  - ✓ Denaturation 95 °C, 15 seconds
  - ✓ Annealing- Extension 60°C, 30 seconds
  - ✓ Cycle number 40
  - ✓ Analysis time 60 minutes

Gene	Detector
Total Cyanobacteria (16S rDNA)	FAM (495/516nm)
Internal Amplification Control (IAC)	Cy3/ CalFluor Orange (CFO - 538/559nm)
Microcystin/Nodularin (mcyE/ndaF	FAM (495/516nm)
Cylindrospermospin (cyrA)	Cy3/CalFluor Orange (CFO - 538/559nm)
Saxitoxin (Sxt A)	TxR/CalFluor Red (CFR - 590/610nm)

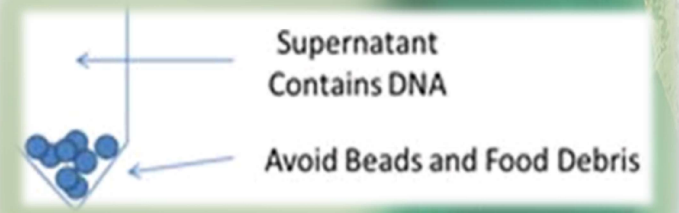
qPCR Workflow



Sample Preparation

- ✓ Direct Sample Analysis:
  - ✓ Transfer 500ul of a mixed sample to a BioGX Bead Lysis Tube, bead beat for 1.5 minutes, centrifuge to pellet the beads and cells, analyze the supernatant

- ✓ ***Filtration***
  - ✓ Filter 1ml to 100ml through a 0.8 micron polycarbonate filter
  - ✓ Place the filter in a BioGX Bead Lysis Tube, bead beat for 1.5 minutes, followed by two cycles centrifugation, analyze the supernatant

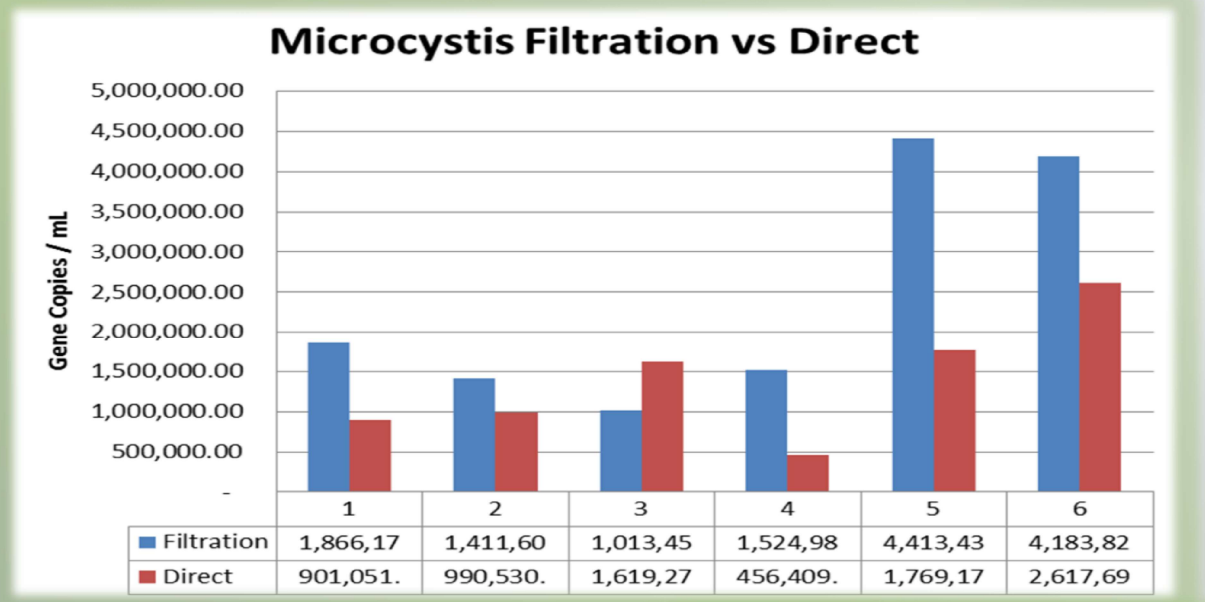


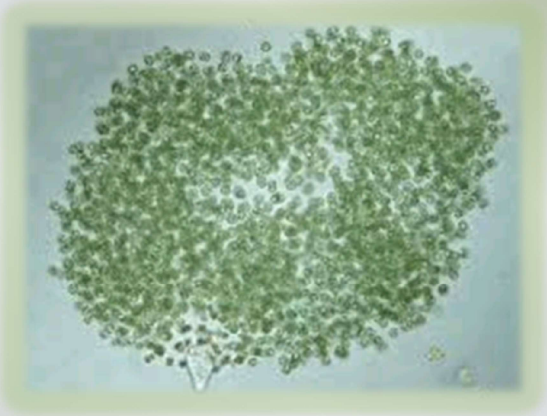
- ✓ Centrifugation
  - ✓ Centrifuge 10ml of sample for at least 20 minutes and pour off supernatant
  - ✓ Resuspend pellet cells in 400ul of lysis buffer, transfer the suspension to a BioGX Bead Lysis Tube, bead beat, analyze the supernatant

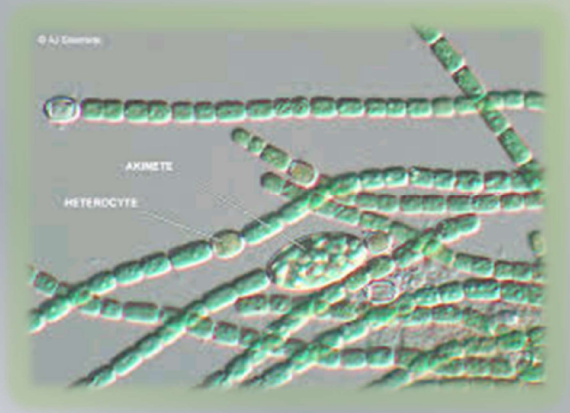
Why Filtration?

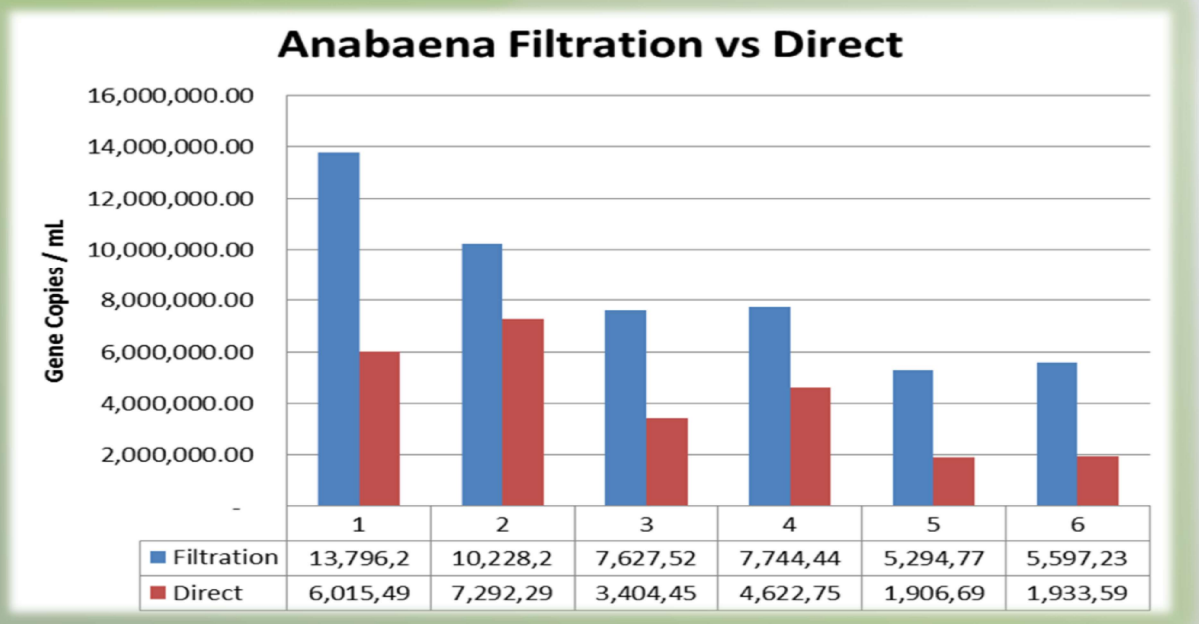
- ✓ Similar methodology to EPA qPCR methods
- ✓ Multiple filter sizes and apparatus available
  - ✓ 0.8 μM pore size – chosen based on cyanobacterial cell size
  - ✓ 45 mm surface area of filtration base decreases filtration time
  - ✓ Membrane filtration apparatus readily available
  - ✓ Filtration funnels are sterile and disposable
  - ✓ Less time to filter than syringe filtration
  - ✓ Greater recovery than centrifugation and direct sample analysis

Why Filtration?



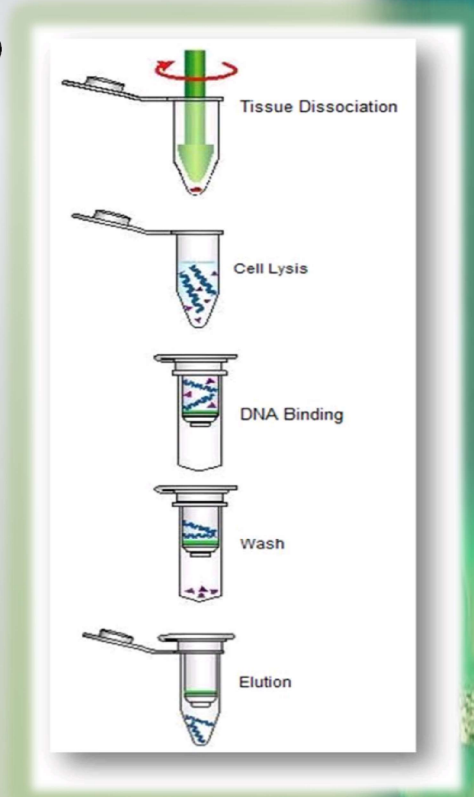


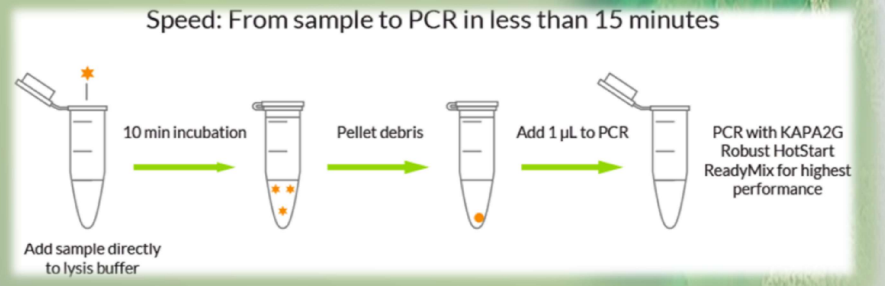




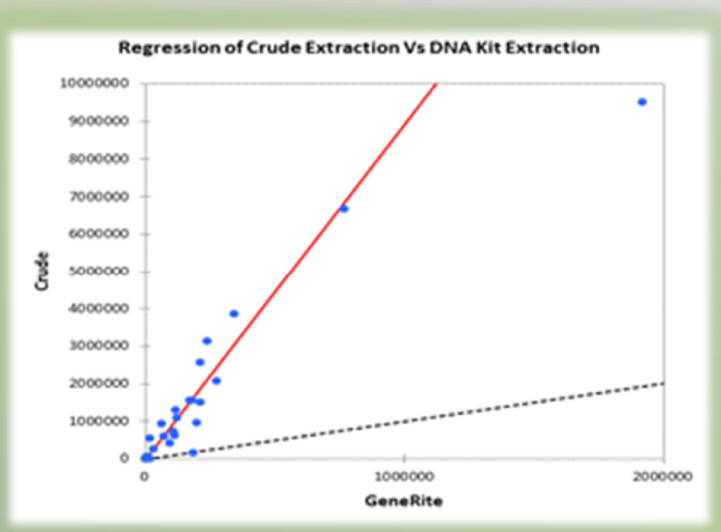
To Purify or Not to Purify

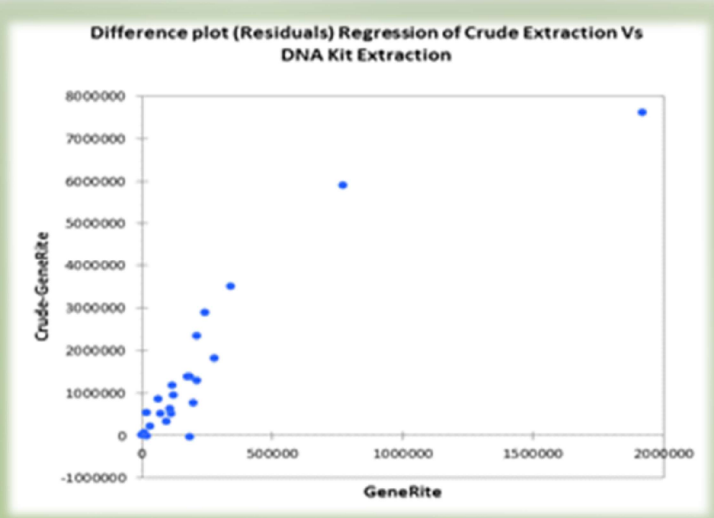
- ✓ DNA extraction kits are used to purify and concentrate DNA and remove inhibitory compounds
  - ✓ Additional steps and reagents
  - ✓ Increase of processing time
  - ✓ Most purification kits begin with a crude extraction procedure
- ✓ Crude Extraction
  - ✓ Simplified extraction
    - ✓ Filter, cell disruption and centrifugation

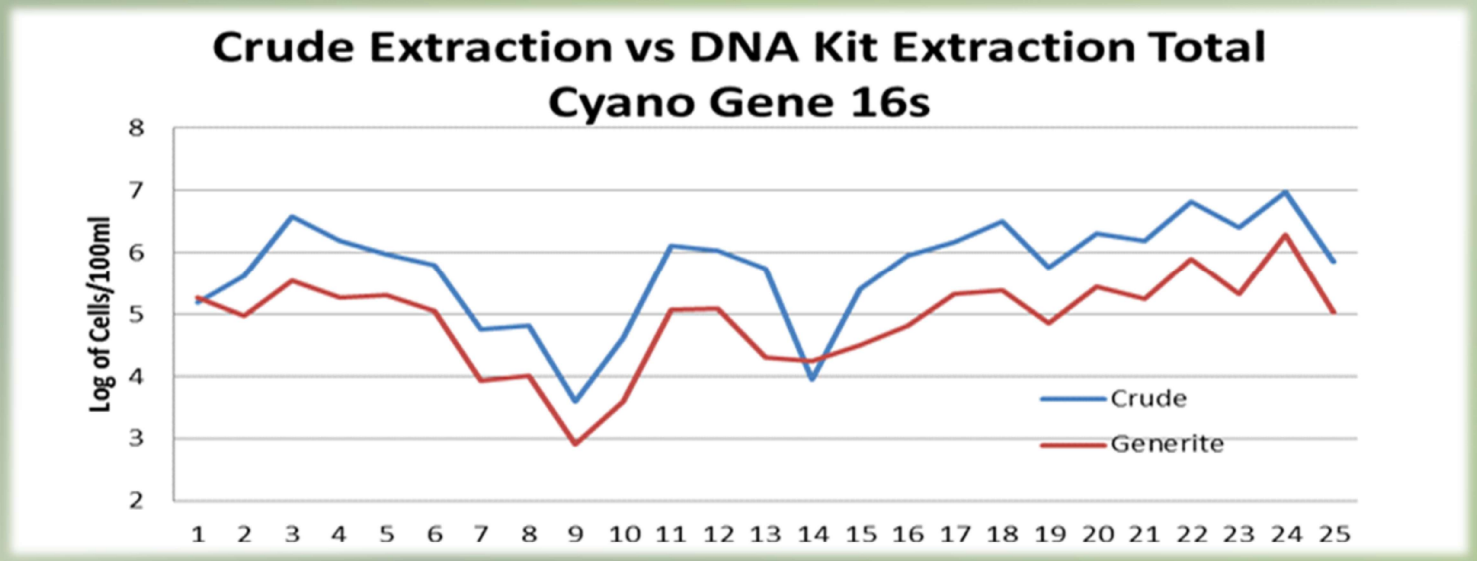




DNA Extraction Comparison





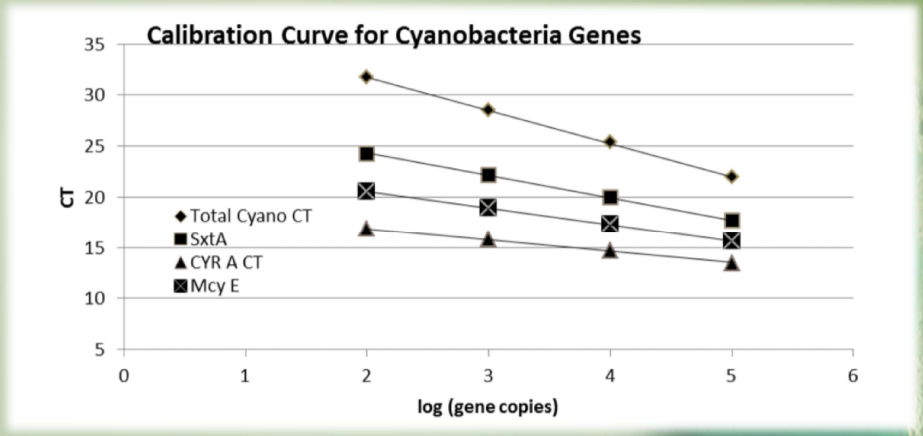


LAKE SUPERIOR STATE UNIVERSITY

WAYNE STATE

Calibration

- ✓ CyanoNAS Standard Kit
  - ✓ Calibration Methods 2 choices
    - ✓ Use all six standards individually
    - ✓ Perform a serial dilution of NA026
      - ✓ Use other standards as QC Checks



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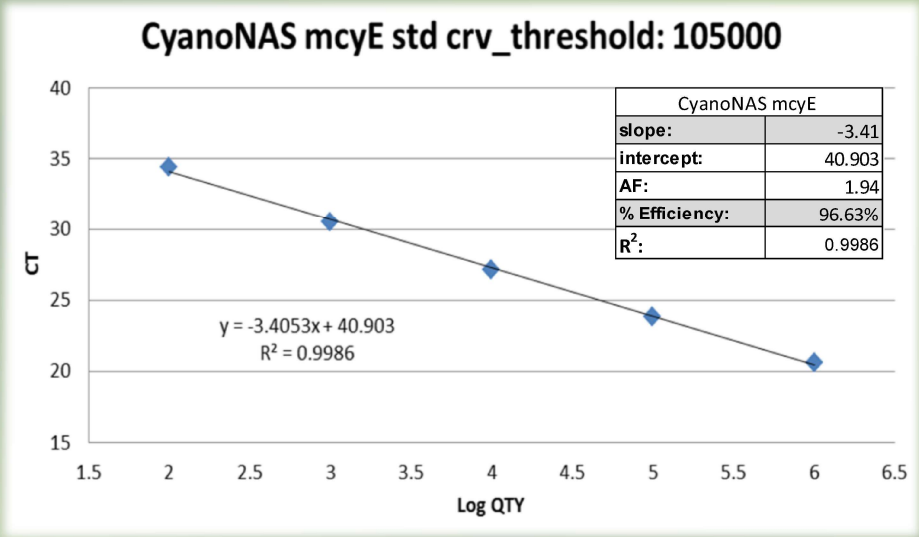
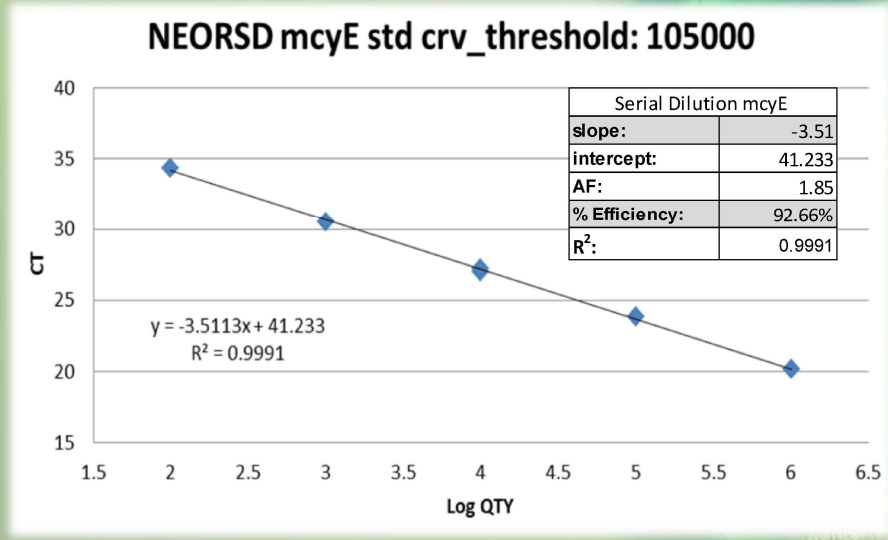
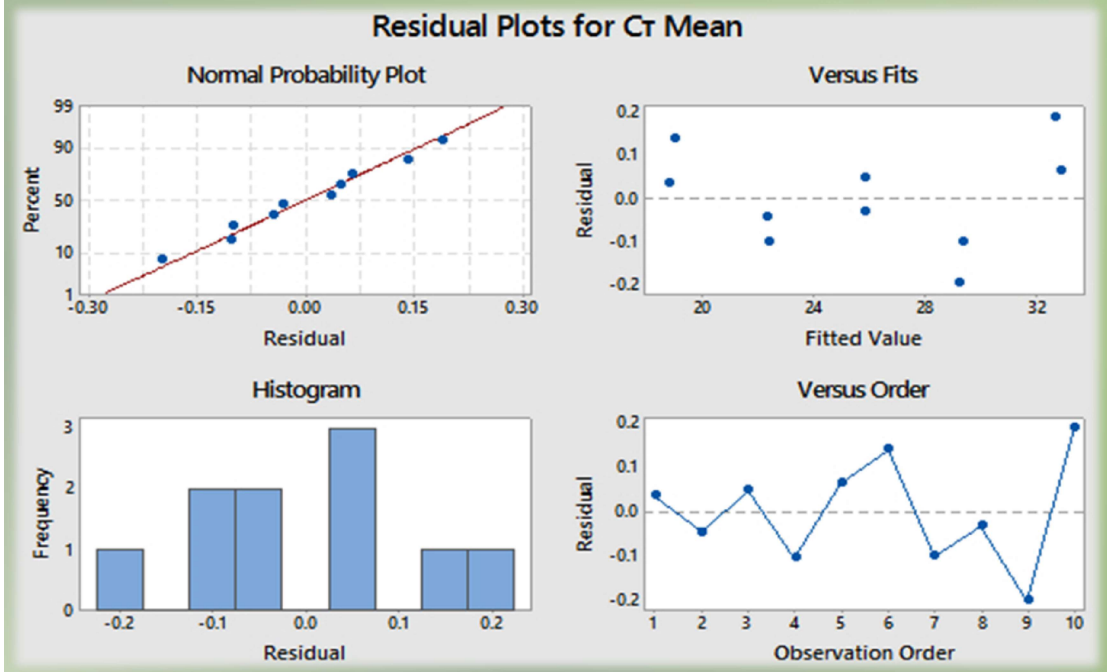
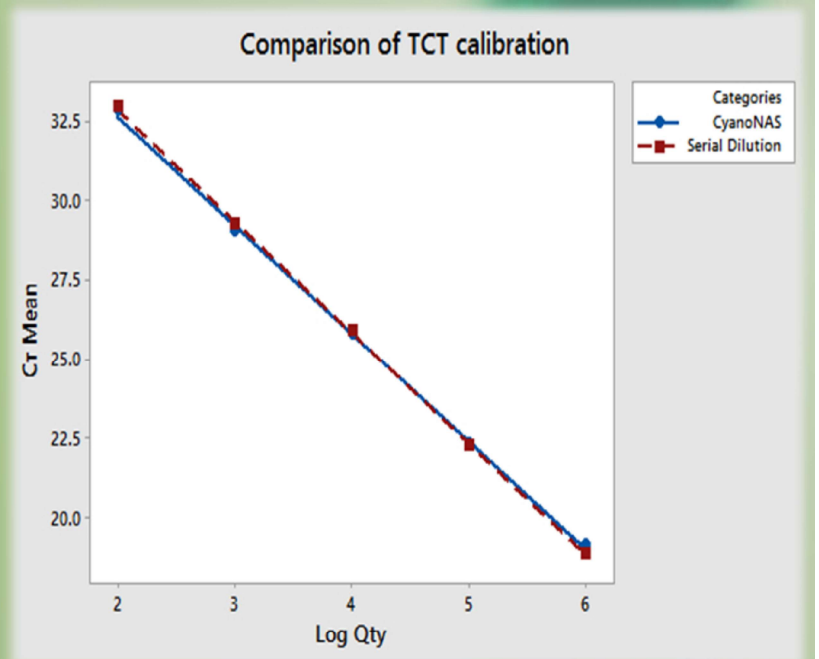




Calibration Comparison

- Calibrating the instrument with the standards from the kit or serial dilution are identical
  - Prefer the serial dilution and use the two standards as check standards

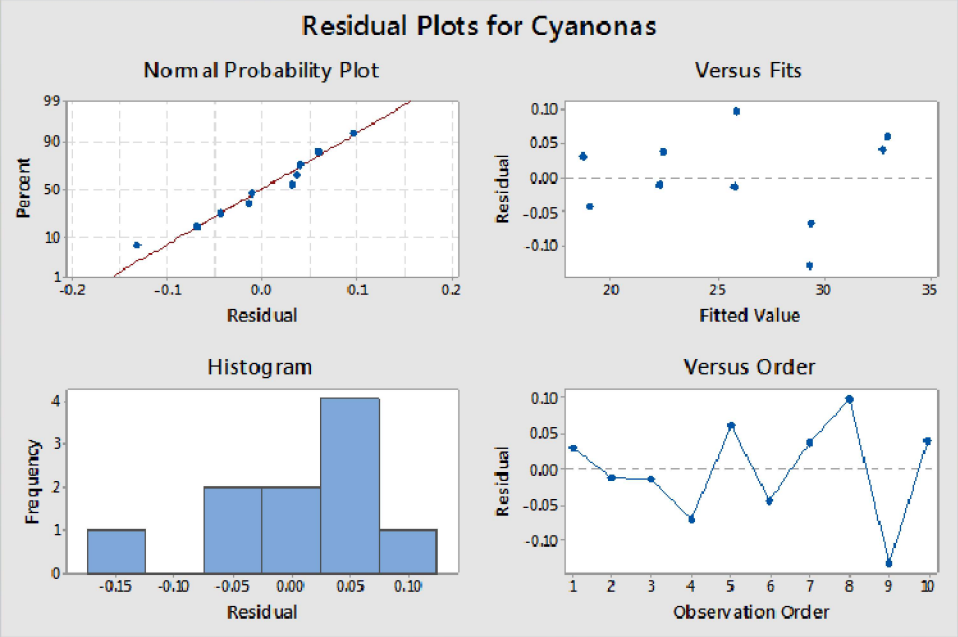


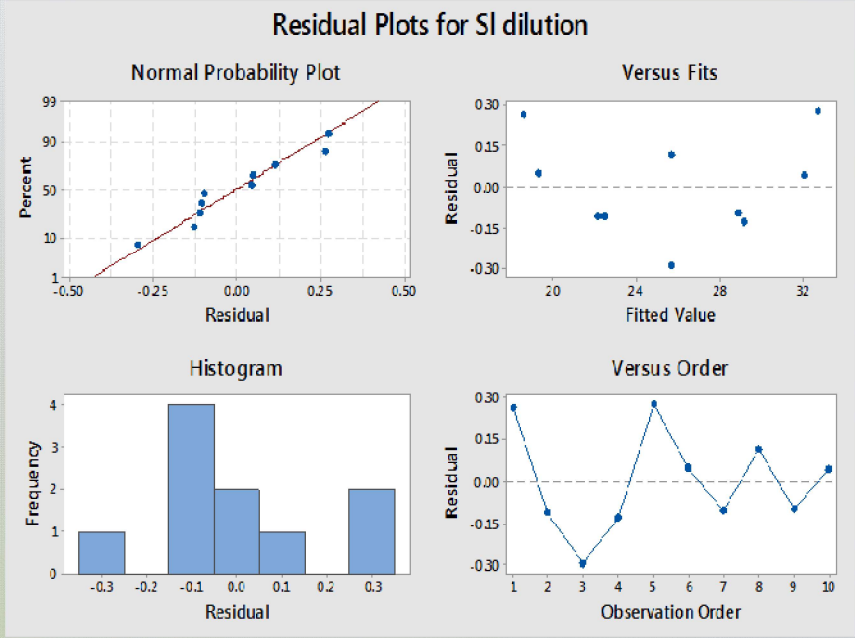


Method Validation Calibration

Serial Dilution Compared to CyanoNAS								
Entity	Calibration Date	Gene	Slope-SD	Slope CyanoNAS	P-Value	Intercept-SD	Intercept CyanoNAS	P-Value
Lab 1	4/20/2016	Total Cyano 16s sRNA	-3.523	-3.423	0.174	39.496	39.910	0.184
Lab 1	4/20/2016	mcyE	-3.511	-3.426	0.389	41.246	41.006	0.561
Lab 1	4/20/2016	Sxt A	-3.387	-3.385	0.978	39.493	39.518	0.941
Lab 1	4/20/2016	cryA	3.373	-3.340	0.531	40.208	40.074	0.553
Lab 2	4/26/2016	Total Cyano 16s sRNA	-3.533	-3.575	0.671	39.794	40.155	0.406
Lab 2	4/26/2016	mcyE	-3.563	-3.433	0.168	41.729	41.592	0.709
Lab 2	4/26/2016	Sxt A	-3.328	-3.310	0.811	39.459	39.438	0.949
Lab 2	4/26/2016	cryA	-3.311	-3.338	0.622	40.049	40.400	0.158
Lab 2	5/5/2016	Total Cyano 16s sRNA	-3.205	-3.437	0.003	38.521	39.639	0.002
Lab 2	5/5/2016	mcyE	-3.298	-3.395	0.196	40.0431	41.416	0.041
Lab 2	5/5/2016	Sxt A	-3.332	-3.229	0.159	39.181	38.763	0.176
Lab 2	5/5/2016	cryA	-3.203	-3.281	0.015	39.481	39.988	0.071

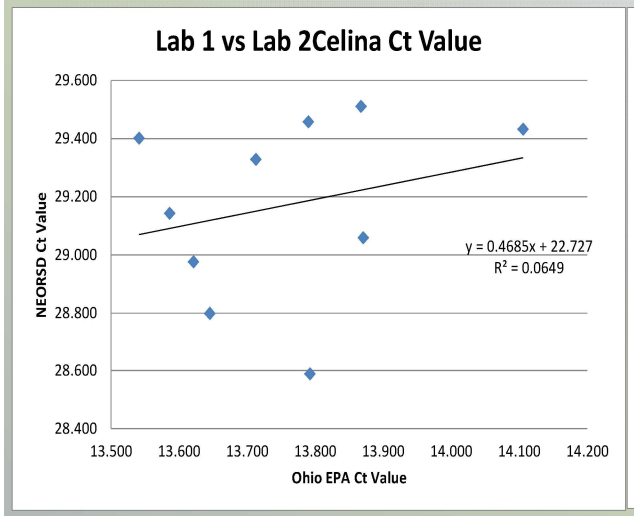
Method Validation Calibration

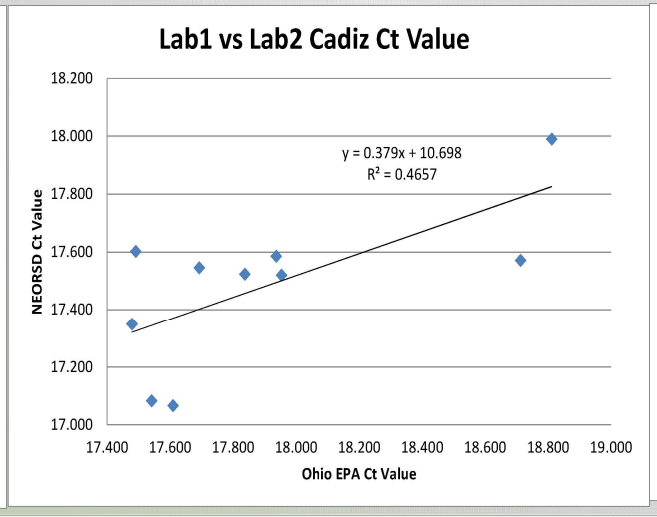


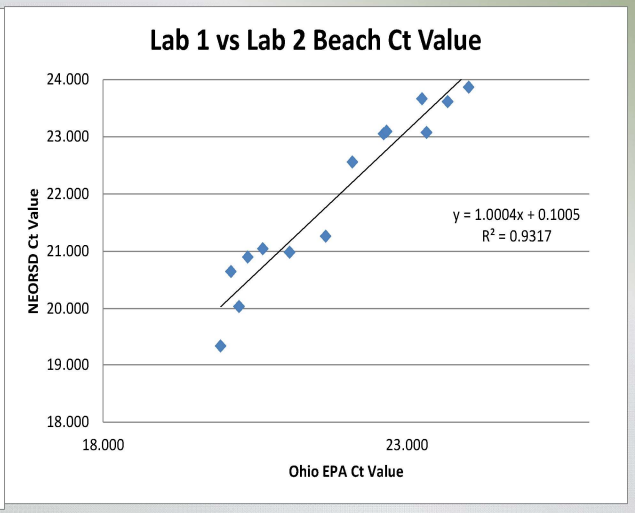


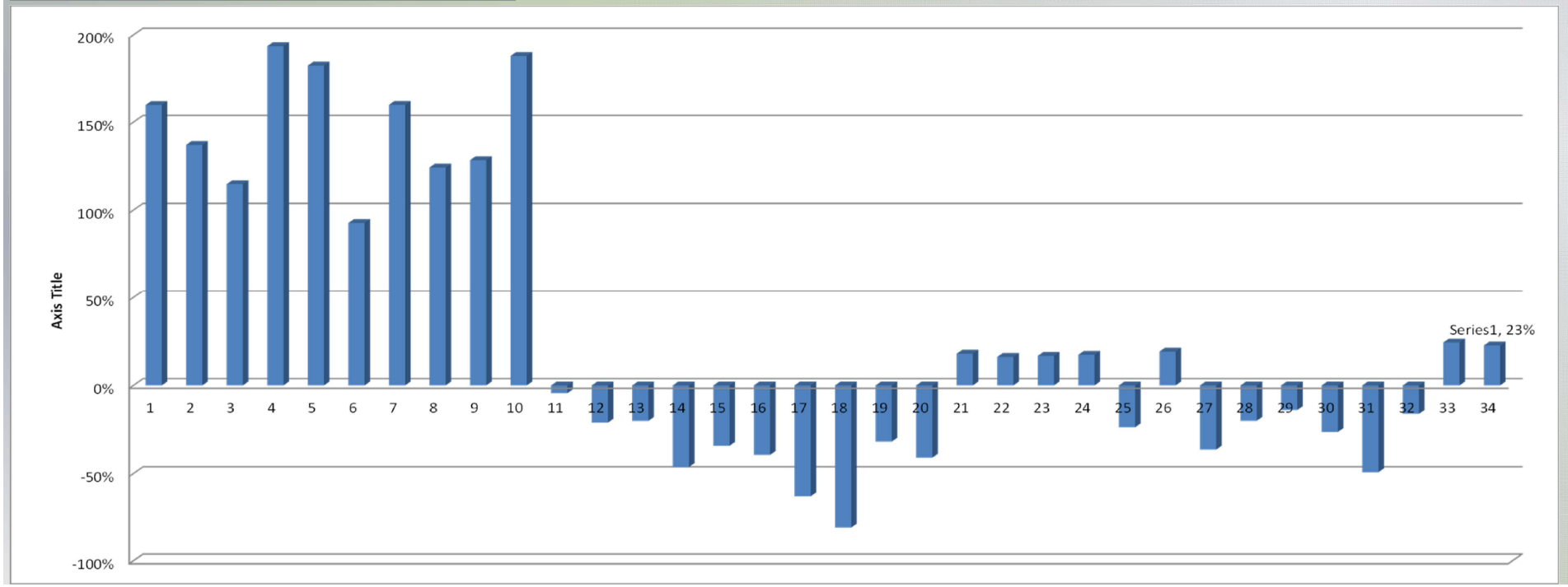
	NEORSD Lab and Second Laboratory					CyanoDetect		
Standard	Average	Max	Min	Stdev	%RSD	Ct	SD	%RDS
200,000 copies	18.963	19.340	18.660	0.206	1.09%	18.890	0.160	0.85%
20,000 copies	22.277	22.507	21.960	0.174	0.78%	22.340	0.060	0.27%
2,000 copies	25.795	26.050	25.370	0.176	0.68%	25.890	0.130	0.50%
200 copies	29.123	29.360	28.810	0.180	0.62%	29.260	0.090	0.31%
20 copies	32.832	33.176	32.150	0.291	0.89%	32.830	0.180	0.55%

Sample Comparison





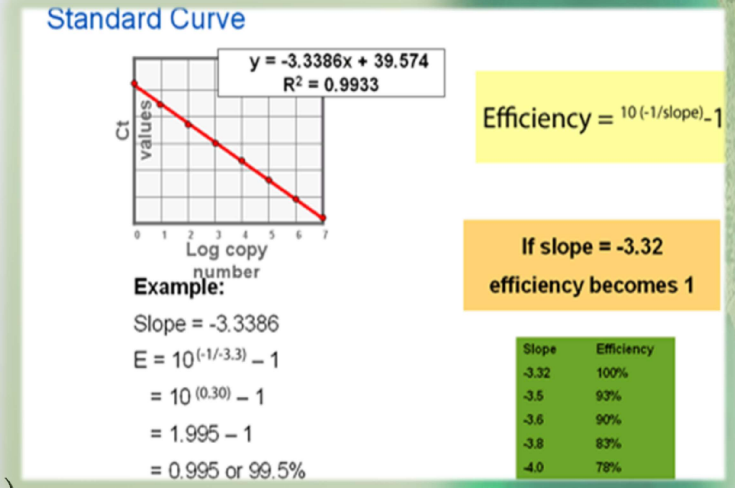


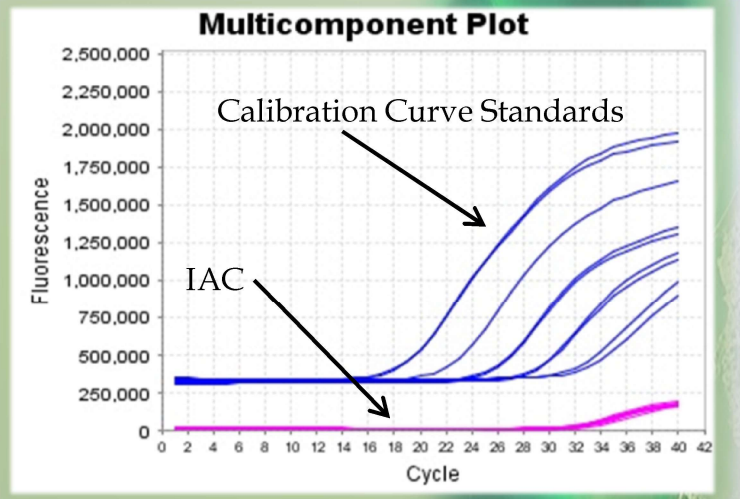


Method Quality Control

- ✓ This method was designed as a screening method and not as an official EPA method.
- ✓ Current Method QC
  - ✓ Correlation $R^2 > 0.995$
  - ✓ Efficiency between 90–100% ($-3.6 \geq$ slope ≥ -3.3)
  - ✓ IAC Internal Amplification Control
  - ✓ Mixed DNA Standard Certified Values
  - ✓ Check Standard
    - ✓ 10,000 and mixed CyanoGene standard

CyanoNAS mcyE	
slope:	3.51
intercept:	41.233
AF:	1.85
% Efficiency:	92.66%
R^2:	0.9991



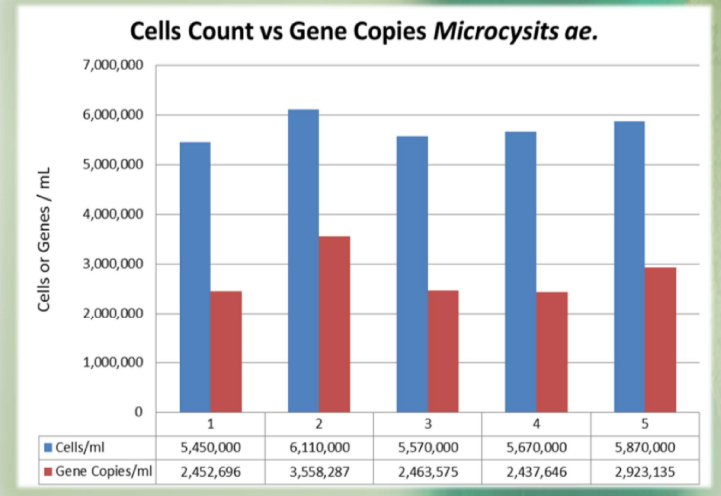


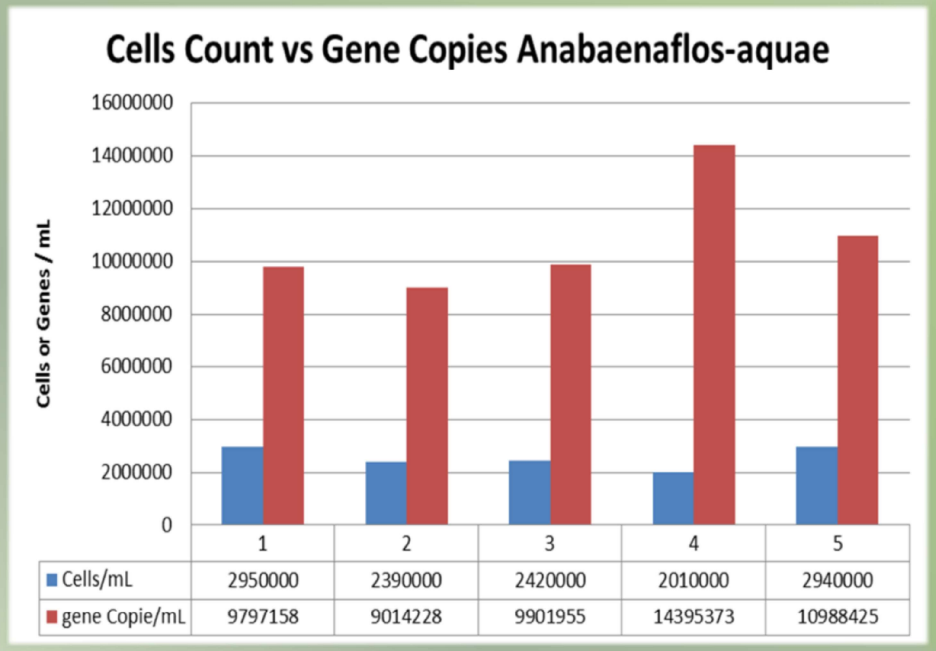
Additional Quality Control

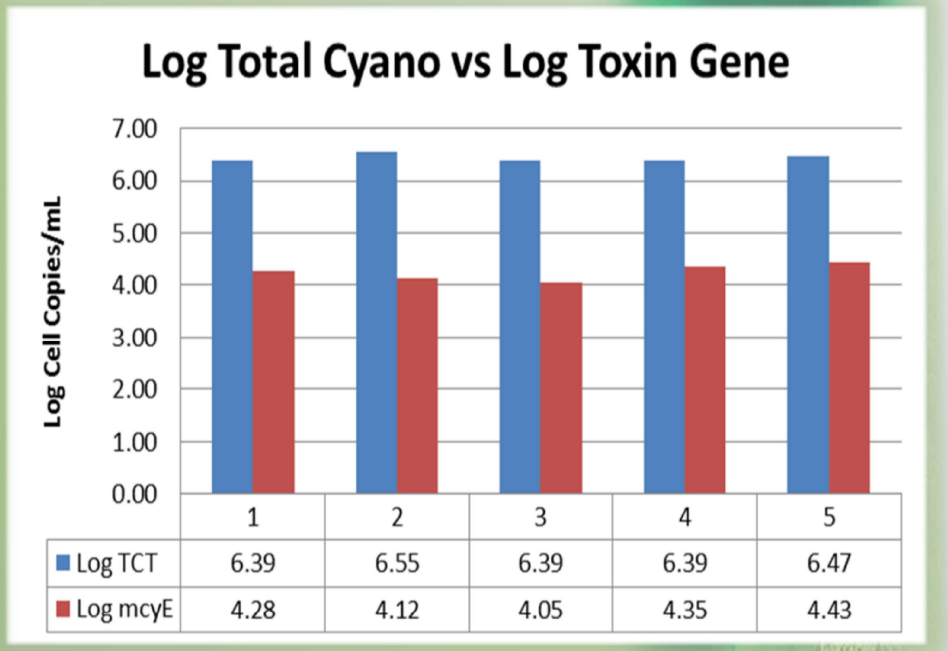
- ✓ USEP Method 1609 and 1611 Analysis of Enterococci by qPCR requires some additional quality control parameters
  - ✓ Calibrator (Continuing Check)
  - ✓ SPC Sample Processing Control (SKETA)
  - ✓ Laboratory Control Standards
    - ✓ Laboratory Control Blank – Reagent water processed through all steps of the method
    - ✓ Laboratory Control Standard – Standard process through all steps of the method
- ✓ Independent Check Standard – Check standard from a different lot
- ✓ With increased QA/QC
  - ✓ Increase of data quality and validity
  - ✓ Increase in analysis cost and time
- ✓ This assay is a screening tool and the cost to add additional QC samples could outweigh the benefits of cost and time savings

Pure Culture

- ✓ Pure cyanobacteria cultures from the Canadian Phycological Culture Center (CPCC)
  - ✓ Microcystis aeruginosa CPCC #299
  - ✓ Anabaena flos-aquae CPCC #67 (filamentous)

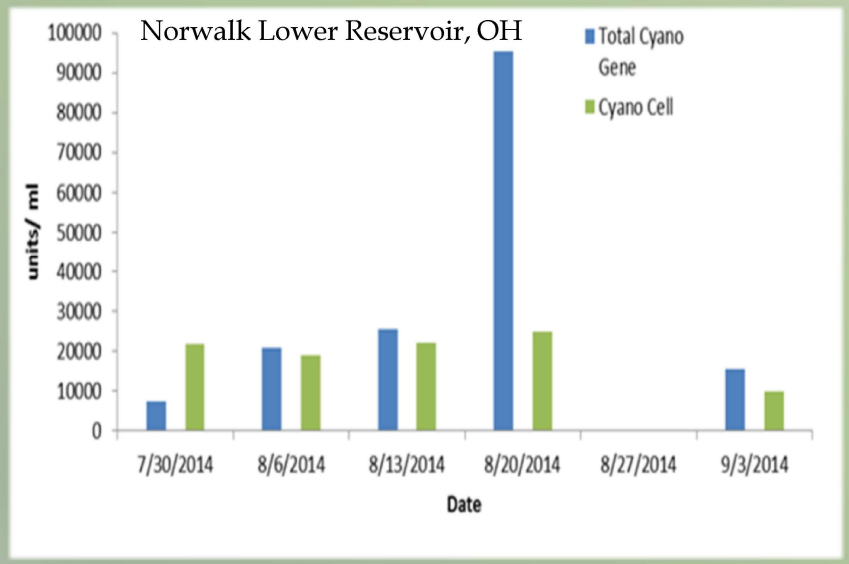


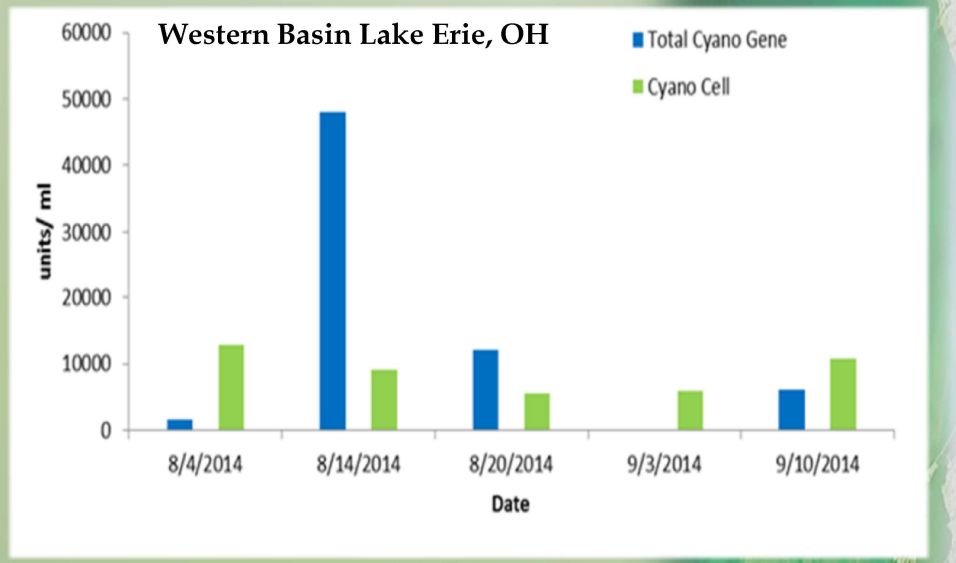




Algae Counts vs Gene Counts

- Comparative study using qPCR and cell counts
  - NEORSD performed the qPCR work

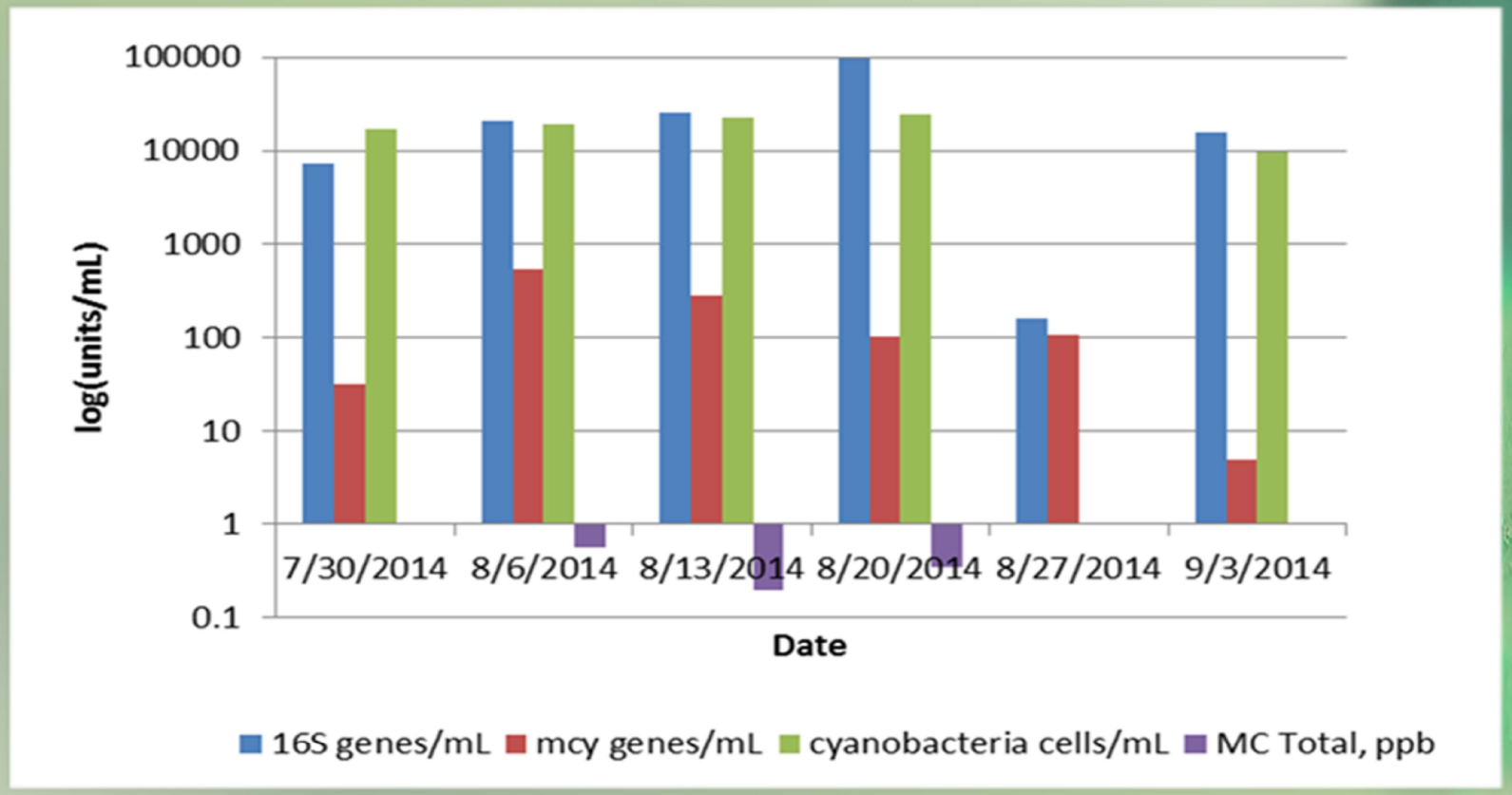




Judy Westrick PhD, Paul Zimba PhD, David Szlag, PhD, Benjamin Southwell, MS

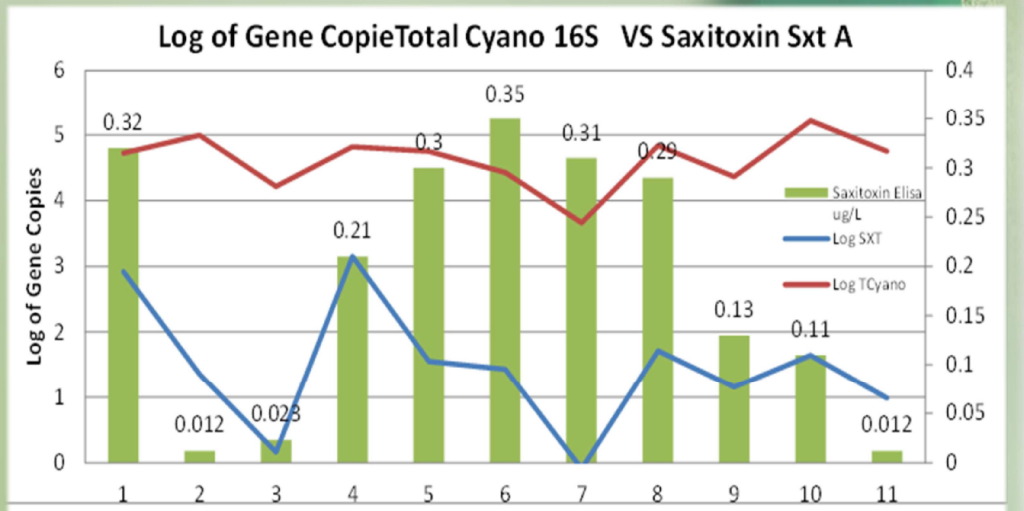
Comparison

- The samples were collected from the Western Basin of Lake Erie for enumeration, toxin analysis and qPCR analysis



Reservoir Profile Results

- A reservoir profile perform in Ohio
  - Saxitoxin ug/L
  - Total Cyano Gene
  - Saxitoxin Gene
- Indiana Department of Environmental Monitoring (IDEM)
  - Similar study in 2016



Sample Location	Collection Date	Sxta qPCR Gene Copies/mL	Saxitoxin Elisa ug/L	T-Cyano 16SRNA Copies/mL
Boat Ramp	8/20/2015	840.2	0.32	53179
Wetland - 1	8/20/2015	22.7	0.012	99812
Wetland - 2	8/20/2015	1.4	0.023	16455
West Bank	8/20/2015	1427.7	0.21	66088
New Intake Photic - Top	8/26/2015	35.5	0.3	56534
New Intake Benthic - Bottom	8/26/2015	27.0	0.35	27067
Old Intake	8/26/2015	0.8	0.31	4609
L2 Photic - Top	8/26/2015	51.8	0.29	69471
L2 Benthic - Bottom	8/26/2015	14.3	0.13	23162
L3 Photic - Top	8/26/2015	44.2	0.11	165159
L3 Benthic - Bottom	8/26/2015	9.6	0.012	56582
L4 Column	8/26/2015	<1.0	<0.05	2814

Conclusion

- Assay appears to be promising however additional data is needed for evaluation
- Inter-laboratory validation study
- Ohio EPA collecting samples 2 x a month 2016
- 2016 NEORSD project with some local PWS
  - ELISA, qPCR, LC/MS/MS
  - Add to beach monitoring activities
- 2016 Reservoir Monitoring Project
  - Indiana DEM

Questions

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