Biotoxin Gene qPCR Assays For The Monitoring And Management Of Biotoxin Risk In Aquatic Environments

Greg Ford
Phytoxigene
Blooms occurring across North America

Owasco Lake, New York, 2017

L. Okeechobee, Florida, 2016

Detroit Lake, Oregon, 2018

Lake Erie, Ohio, 2017
Biotoxins, Water Quality and Health

• Toxic compounds are biosynthetically produced secondary metabolites with little understanding to why and specifically when they may occur.

• Increasing in frequency, severity
Biotoxins, Water Quality and Health

Dangers:

- Skin rashes, allergic reactions
- Neurologic effects
- Liver damage
- Genotoxic
- Tumor promoting
- Death
"Our emergency rooms saw an increase in a range of symptoms that included nausea, vomiting, skin rashes, coughing, shortness of breath."

**Dr. Steve Parr**, Director of Emergency Medicine Martin Health Systems, on the 2016 Algae Blooms in Martin County, Florida.

Biotoxins, Water Quality and Health

Blue-green algae in St. Lucie River sending people to emergency rooms
Treasure Coast Newspapers, July 26, 2018

Scientists have discovered a link between blooms of cyanobacteria - blue-green algae - like the kind seen on lakes and rivers in Florida - with Alzheimers and ALS disease. ED KILLER/TCPALM Wochit

Fifteen people treated Monday and Tuesday by Martin Health System emergency rooms, clinics and primary care doctors for symptoms consistent with algae toxins reported they had contact with the St. Lucie River within the previous seven days.
“In fact, the death rate from liver disease not related to alcohol was nearly twice as high in the four (Florida) counties (Martin, St. Lucie, Indian River, Okeechobee) as the national rate during the 12 years of the OSU study, according to data calculated for TCPalm by the Centers for Disease Control and Prevention.”

TC Palm News, May 22, 2017
Biotoxins, Water Quality and Health

Human fatalities from cyanobacteria: chemical and biological evidence for cyanotoxins.


Abstract

An outbreak of acute liver failure occurred at a dialysis center in Caruaru, Brazil (8 degrees 17' S, 35 degrees 58' W), 134 km from Recife, the state capital of Pernambuco. At the clinic, 116 (89%) of 131 patients experienced visual disturbances, nausea, and vomiting after routine hemodialysis treatment on 13-20 February 1996. Subsequently, 100 patients developed acute liver failure, and of these 76 died. As of
### 2017 EPA Guidance, HABS

**EPA Guidance for drinking and recreational waters (draft)**

<table>
<thead>
<tr>
<th>Cyanotoxin</th>
<th>Drinking Water Health Advisory (10-day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bottle-fed infants and pre-school children</td>
</tr>
<tr>
<td>Microsystins</td>
<td>0.3 μg/L</td>
</tr>
<tr>
<td>Cylindrospermopsin</td>
<td>0.7 μg/L</td>
</tr>
</tbody>
</table>

**Draft recreational water guidance:**
- Swimming advisory: not to be exceeded on any day
- Recreational criteria for waterbody impairment: not exceeded more than 10% of days per recreational season up to 1 calendar year.

<table>
<thead>
<tr>
<th>Microcystins</th>
<th>Cylindrospermopsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 μg/L</td>
<td>8 μg/L</td>
</tr>
</tbody>
</table>
Tools to Measure Toxins

<table>
<thead>
<tr>
<th>Biologica Assays (Class Specific Methods at Best):</th>
<th>Anatoxins</th>
<th>Cylindrospermopsins</th>
<th>Microcystins</th>
<th>Nodularins</th>
<th>Saxitoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>PPxA</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Neurochemical</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>ELISA</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chromatographic Methods (Compound Specific Methods):</th>
<th>Freshwater Cyanotoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gas Chromatography:</strong></td>
<td>Anatoxins</td>
</tr>
<tr>
<td>GC/FID</td>
<td>Yes</td>
</tr>
<tr>
<td>GC/MS</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Liquid Chromatography:</strong></th>
<th>Anatoxins</th>
<th>Cylindrospermopsins</th>
<th>Microcystins</th>
<th>Nodularins</th>
<th>Saxitoxins</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC/UV (or HPLC)</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>LC/FL</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

*Liquid chromatography combined with mass spectrometry can analyze cyanotoxins very specifically.*

| LC/IT MS                                             | Yes       | Yes                 | Yes          | Yes        | Yes       |
| LC/TOF MS                                            | Yes       | Yes                 | Yes          | Yes        | Yes       |
| LC/MS                                                | Yes       | Yes                 | Yes          | Yes        | Yes       |
| LC/MS/MS/MS                                          | Yes       | Yes                 | Yes          | Yes        | Yes       |

Tools to Screen Samples

**Enumeration** (species identification by microscope, cell counting)
- Requires special training.
- More subjective
- Time consuming
- Does not confirm which toxin or toxin production

**Flow Cytometry**
- Addresses some of the drawbacks of manual enumeration
- Higher Capital Investment
- Does not confirm which toxin or toxin production

**Fluorometer**  Indicates presence & relative amount of total Cyanobacteria by measuring phycocyanin.
- Low operational cost, nominal capital investment
- Does not confirm which toxin or toxin production

**Genetic/qPCR**  Measures gene copies for total Cyanobacteria and gene copies for specific toxins.
- Toxin specific
- ~Two-Three Hours to answer
- Demonstrated predictive capability, demonstrated correlation to toxin production
CyanoDTec; Genetic Approach

First Standardised cyanobacterial toxin gene assay
Feasible to monitor and test more frequently
Predictive
More sensitive than current morphological methods
Non species dependent
Rapid turnaround of results
Reduce need/frequency of higher cost taxonomic identification and analytical methods, only confirm when toxin gene count reaches threshold
The Phytoxigene test is based on simple paradigm;

Like any biological product, toxins have a genetic pathway, if the gene is present then toxin can be produced.
The Phytoxigene test is based on simple paradigm; *Like any biological product, toxins have a genetic pathway, if the gene is present then toxin can be produced.*

From gene to toxin...microcystin

Conversely if gene not present, no toxin can be produced
qPCR Reaction
Components

• Water
• DNA template
• Primers/Probes*
• Nucleotides*
• Mg++ ions *
• DNA Polymerase*

*Reagents lyophilized into CyanoDTec Assay. Just add water.
National Measurement Institute

Mandate

- Develop primary measurement methods, reference materials and infrastructure to allow traceable measurements to be performed across all areas of science.

Development of CyanoDTec Standards
Northeast Ohio Regional Sewer District 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.

<table>
<thead>
<tr>
<th>Cyanobacterial Genera</th>
<th>Hepatotoxins</th>
<th>Neurotoxin</th>
<th>Tastes and Odors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYLINDROSPERMOPSIS</td>
<td>MICROCYSTINS</td>
<td>ANATOXIN</td>
</tr>
<tr>
<td>Anabaena</td>
<td>X</td>
<td></td>
<td>X</td>
</tr>
<tr>
<td>Anabaenopsis</td>
<td>X</td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Aphanizomenon</td>
<td></td>
<td>X</td>
<td></td>
</tr>
<tr>
<td>Aphanocapsa</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cylindrospermopsis</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Methods for Toxin Analysis

Most laboratories use the ELISA method to quantify the following cyanotoxins: cylindrospermopsin, 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
Lake Okeechobee has experienced harmful algal blooms since the 1980s

- Largest lake in the southeast USA
- Shallow (ave. depth 2.7m), impacted by agricultural runoff, toxic blooms observed
- Water levels regulated by USACE to protect the integrity of the Herbert Hoover Dike
- Long-term dataset (15+ years; Karl Havens / South Florida Water Management District)

Photo: Joshua Stevens—NASA Earth Observatory
Have been dominated by *Planktothrix*, *Oscillatoria* or *Microcystis*

This and following slide courtesy of Dr. Tim Davis, Bowling Green State University
*mcyE* copy number correlates with MCs and total N

Kramer et al., 2018, PLoS ONE, 13(5): e0196278
Best validation of the technology, well over 2,000 samples run. Side-by-side comparison to microcystin toxin testing (1850 sample data set)

Link to 2017 Ohio EPA update:
http://epa.ohio.gov/Portals/28/documents/habs/4-4-17_HAB_Webinar.pdf
Current Monitoring Requirements

**Total Microcystins**
- May-October *(new reduced options)*
  - Weekly raw/finished water
- November-April
  - Biweekly raw water only
- If purchasing SW from out of state
  - Weekly finished water microcystins monitoring required (year round)

**Cyanobacteria Screening (qPCR)**
- Biweekly raw water
- Triggers follow up sampling by Ohio EPA for other cyanotoxins
qPCR as Screening Tool for Microcystins

- Microcystins detected in raw water at 45 PWSs (38%) and mcyE detected at 57 PWSs (48%)
  - mcyE detections at all 25 Lake Erie PWSs
  - Microcystins detections at 22 Lake Erie PWSs
- Out of 1850 paired PWS samples:
  - 100% of microcystins >1.6 µg/L had paired mcyE gene detections.
  - 100% of microcystins >5 µg/L had mcyE detections > 5gc/µL
  - 90% of microcystins detections >1.6 ug/L had mcyE detections >5 gc/µL
  - Less than 2% of samples (22 sites, 32 samples) had microcystins detections without mcyE detections:
    - 19 of the 22 sites had gene detections in either prior or post sampling events
    - The remaining three PWSs had only one low level (0.35 – 0.44 µg/L) microcystins detection in 2016; all had trace mcyE gene copies
Example: Phytoplankton Enumeration versus qPCR
qPCR as Screening Tool for Microcystins

2016: Out of

- 100% of microcystins detections greater than 1.6 ug/L had paired mcyE gene detections.
- Only 2% of samples had microcystins detections without corresponding gene detections:
  - 22 PWSs had at least one MC detection without corresponding mcyE detection, but 19 of the 22 had gene detections in either prior or post-sampling events.
  - The remaining three PWSs had only one low-level (0.35 – 0.44 ug/L) microcystins detection this season. Two of those systems had trace mcyE gene copies (detected, but below DES reporting limit).
- Overall, qPCR is a reliable screen for Microcystins.

![Microcystins and qPCR Trends](image-url)
Example: Source Water With Consistently High Microcystins & mcyE Concentrations

![Graph showing Microcystins (µg/L) and mcyE gene (gc/µL) concentrations over time from 6/1/16 to 12/18/16.](image-url)
Example: Simultaneous Saxitoxins and Microcystins

Microcystins, mcyE

Saxitoxins, sxtA, 16S

ND

5/30/16 7/19/16 9/7/16 10/27/16 12/16/16

Microcystins (µg/L)  mcyE (GC/µL)  16S (GC/µL)

Saxitoxins (µg/L)  sxtA (GC/µL)
Inland Lake Examples: Saxitoxin-producing Cyanobacteria, sxtA, and Saxitoxins

![Graph showing cyanobacteria and saxitoxin levels in different sites.](https://via.placeholder.com/150)

- **Cyanobacteria (cells/µL)**
- **Saxitoxins (µg/L)** & **sxtA (GC/µL)**

- **Sites**:
  - A
  - B
  - C
  - D1
  - D2
  - D3
  - D4
  - E1
  - E2
  - F

- **Cyanobacteria Species**:
  - *Cylindrospermopsis*
  - *Aphanizomenon*
  - *Dolichospermum (Anabaena)*

- **Markers**:
  - Red dot: Saxitoxins
  - Blue bars: Cyanobacteria
  - Orange bars: *Dolichospermum (Anabaena)*

**Legend**:
- *Cylindrospermopsis*
- *Aphanizomenon*
- *Dolichospermum (Anabaena)*
- Saxitoxins
- Planktothrix
- sxtA
2017 Lake Erie qPCR mcyE Data Summary

- mcyE detections at 22 of 25 PWSs and Microcystins detections at 18 PWSs
  - West Basin: mcyE preceded microcystins at all four water systems by 1-4 weeks.
  - Central Basin (East of Cleveland): mcyE preceded microcystins detections at 6 of 7 PWSs by 1-2 weeks. Trace detection (0.31 ug/L at Painesville not preceded by mcyE).
  - Central Basin (Vermillion – Cleveland): no microcystins
  - Sandusky Subbasin: mcyE preceded microcystins at half (3) PWSs. Trace detections at three PWSs not preceded by mcyE detections (impacted by Sandusky Bay bloom).
Ohio EPA and USEPA Interlab Phytoxigene Assay (16S) Method Comparison

(Samples run by US EPA had been stored for more than one year so some degradation is assumed)
qPCR Data Interpretation

- **mcyE**
  - Any mcyE detection may indicate onset of a bloom, and could provide early warning.
  - More severe blooms, indicated by microcystins concentrations >5µg/L, were always associated with mcyE >5GC/µL.
  - Based on literature, 1.4 gene copies per cell for *Microcystis aeruginosa*.

- **sxtA**
  - sxtA detections were associated with saxitoxins, but in some cases low sxtA was associated with higher saxotoxins concentrations.
  - Based on literature, 2.5 gene copies per cell for *Anabaena circinalis*.

- **16S**
  - 16s detections indicate presence of cyanobacteria, not cyanotoxins.
  - Based on literature, 2.9 - 4.8 gene copies per cell.
Using Molecular Analysis to Direct Reservoir Management

- Saxitoxins Detections in Finished Water from July 31, 2015 – September 21, 2015. Maximum concentration 0.039 ug/L. Maximum raw water concentration at intake 0.812 ug/L

- Extracellular saxitoxins predominated all samples.

- 10 different potential saxitoxin producing genera found in multiple habitat zones (pelagic, benthic, periphyton, etc.) in multiple locations.

- qPCR results indicated benthic saxitoxin source, data used to target algaecide application. Thanks City of Akron PWS & NEORSD!
Sampling Locations
Targeted Algaecide Application Area

23,000 gene copies /ml
<table>
<thead>
<tr>
<th>Date</th>
<th>Location</th>
<th>16s</th>
<th>Mcy</th>
<th>Sxt</th>
</tr>
</thead>
<tbody>
<tr>
<td>7/23/15</td>
<td>Top</td>
<td>637,000</td>
<td>2,000</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Mid</td>
<td>300,000</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>dam</td>
<td>150,000</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>08/11/2015</td>
<td>top</td>
<td>203,000</td>
<td>1,000</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>mid</td>
<td>1170000</td>
<td>9,660</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>dam</td>
<td>557000</td>
<td>1,000</td>
<td>ND</td>
</tr>
<tr>
<td>08/21/2015</td>
<td>mid</td>
<td>273000</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>dam</td>
<td>353000</td>
<td>10,200</td>
<td>ND</td>
</tr>
<tr>
<td>9/17/15</td>
<td>Mid</td>
<td>&gt;2mm</td>
<td>ND</td>
<td>3,780*</td>
</tr>
<tr>
<td>9/25/15</td>
<td>Dam</td>
<td>&gt;2mm</td>
<td>ND</td>
<td>3,460*</td>
</tr>
<tr>
<td>10/06/2015</td>
<td>mid</td>
<td>&gt;2mm</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>dam</td>
<td>&gt;2mm**</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10/14/15</td>
<td>mid</td>
<td>&gt;2mm**</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>Dam</td>
<td>&gt;2mm**</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngya, and Oscillatoria*

**predominantly Planktothrix**
New Zealand drinking water reservoir

- Benthic cyanobacterial mats identified after taste and odour problems reported
- Low levels of toxins detected in mats
- Regular monitoring initiated
New Zealand drinking water reservoir

10 samples collected (c. 20 g) every 2 wks using ponar grab

Homogenise
For 2 mins

Extract DNA using Qiagen PowerSoil kit

Screen samples using Phytoxigene DNA kit, plus in-house anatoxin gene assay

All positive samples confirmed using LC-MS
A positive LC-MS result triggers a series of actions
Use in North America

1. National Level
   • US EPA: Active Validation
   • NOAA: Two Year Study
   • Environment Canada: 2018 Study
   • USGS, Lawrence, KS

2. Regional EPA:
   • Region 7, Kansas City; entering second year of use

3. State EPA/Health Labs
   • Ohio: entering fourth season of use
   • Indiana: entering third season of use
   • Utah: set up, implementing in 2018
   • Idaho: Implementing multiple-reservoirs in 2018
   • Wisconsin: set up, implementing in 2018
   • Oregon; Evaluation, 2019

4. City/Municipal
   • Akron, Northeast Regional Sewer (Cleveland); Ohio
   • Des Moines, IA; Wichita Falls, TX, Grand River (OK)
   • Los Angeles (starting in 2019)

5. Academic & Other Government Research (numerous)

6. Commercial Lab: Alloway Labs (Ohio), North Coast Environmental Labs (Ohio)
Lyophilized Reagent

Steps:
- Filter 10-50ml water sample
- Lysis and release DNA from cells
- Re-hydrolyse bead/enzyme mix in kit
- Add 5ul of sample to 20ul of enzyme mix into reaction rube
- Run PCR

TOTAL time 2-3 hours
Costs/Equipment Needs

Equipment
- Syringe Filter/vacuum pump system
- Vortex Shaker or Bead Beater
- Mini centrifuge
- Real Time PCR system
- Pipettes

Phytoxigene Assay, Per test cost
- $56 for complete Assay, #205-0100

Other Consumables (~$5/test)
- Pipette tips
- Filter paper
- PCR grade water
- Lyse Tubes
Other Assays

DinoDTec for monitoring for Saxitoxin in marine environments, Paralytic Shellfish Poisoning

Microbial contamination in drinking water and recreational water
  Drinking water: Total Coliform and E.coli
  Recreational waters:
    - E.coli & Norovirus in fresh waters
    - Total Enterococcus & Norovirus in marine waters

Parasite Detection in Drinking Water
  Crypto, Giardia, Entamoeba
Crypto Assay

Multiplexed, real-time PCR reagent intended for the *qualitative* detection of Cryptosporidium parvum, Giardia intestinalis, Entamoeba histolytica as well as a Sample Processing Control/Internal Amplification Control (SPC/IAC).

Sample-Ready™ lyophilized

Probable Format: Two kits, three targets each (genus, species, SPC) with identical amplification conditions.

--Format based on results of survey, 31 responses (Greg Sturbaum, Casey Lyon). *Open to input*
ACKNOWLEDGEMENTS

Ohio EPA
Nik Dzamov
Heather Raymond
City of Akron
Jessica Glowczewski