Microcystins: A Lab Perspective

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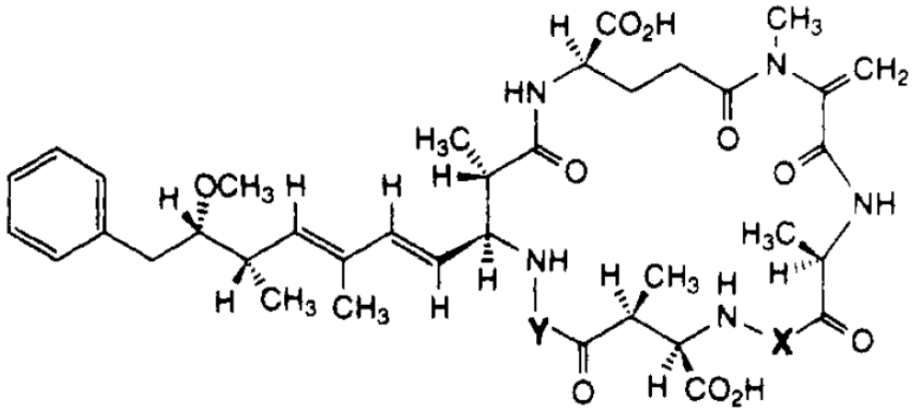
What are microcystin toxins and where do they come from?

Microcystin Toxins

- Produced by *Microcystis sp.*
- Mostly intracellular while alive
- Numerous different congeners (variants)
- Similar in structure to nodularin toxins
  - Found in brackish water
- Peptides composed of amino acids
- LR is the most toxic variant

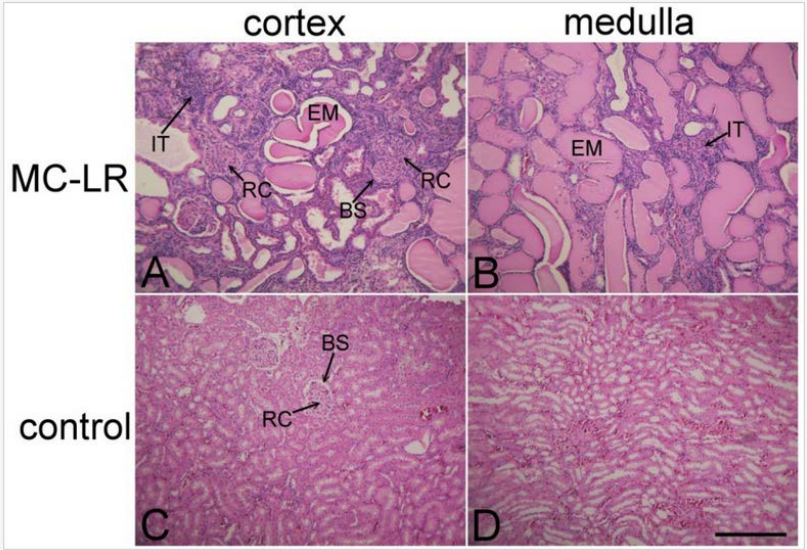


Microcystin Congeners	Amino Acid in X	Amino Acid in Y
Microcystin-LR	Leucine	Arginine
Microcystin-RR	Arginine	Arginine
Microcystin-YR	Tyrosine	Arginine
Microcystin-LA	Leucine	Alanine
Microcystin-LY	Leucine	Tyrosine
Microcystin-LF	Leucine	Phenylalanine
Microcystin-LW	Leucine	Tryptophan

Microcystin Toxins

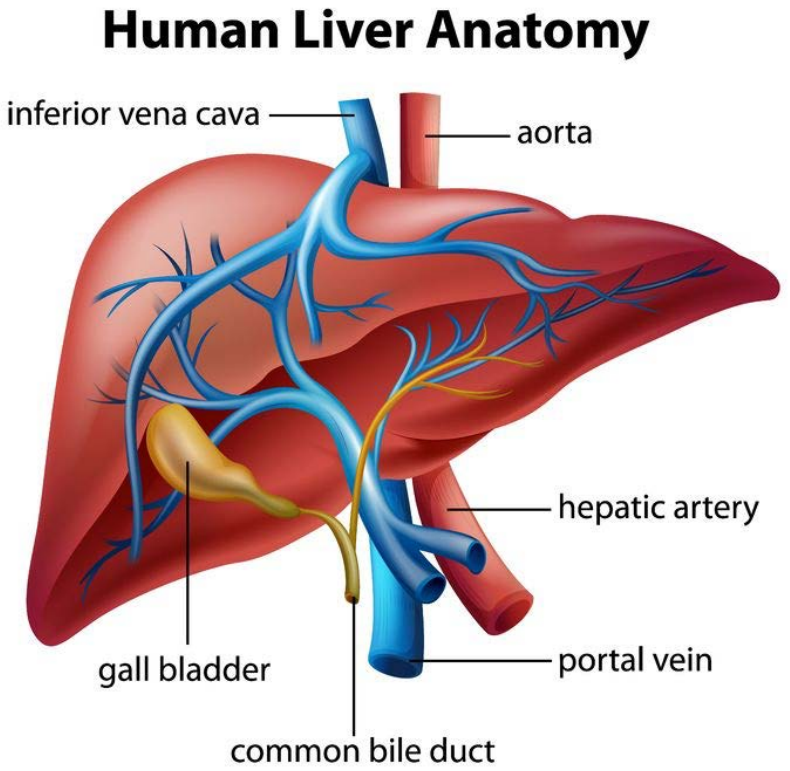
- Potent hepato- and nephrotoxin
- Some GI, heart toxicity observed as well
- Lung tissue lesions were observed in inhalation studies
- Chronic exposures in animal studies
  - Liver tumor promotion
  - Cardiotoxicity
  - Potential mutagen

Figure 3. Nephrotoxic effects of microcystin LR. Kidney slices stained with hematoxylin and eosin in rat kidney cortex (**A**,**C**) and medulla (**B**,**D**). The experimental rat was treated with MC-LR (10 μg/kg i.p.) (**A**,**B**), every other day for eight months. The control rat was treated only with the vehicle (containing 0.8% ethanol, 0.2% methanol and 0.9% NaCl) (**C**,**D**). In the MC-LR treated rat, numerous enlarged renal corpuscles (RC) with compressed Bowman's space (BS) are seen (**A**). The tubules are widened and filled with eosinophilic material (EM), as seen in (**A**,**B**). In-growing interstitial tissue (IT) (**A**,**B**) is infiltrated with mononuclear cells. Normal histological structure of kidney from the control rat is shown in (**C**,**D**). Bar = 300 μm.



Microcystin Toxins

- Clinical symptoms of exposure
  - Abdominal pain
  - Headache
  - Sore throat
  - Vomiting
  - Nausea
  - Dry cough
  - Diarrhea
  - Blistering around the mouth
  - Pneumonia
  - Liver and kidney damage



http://www.fitnea.com/wp-content/uploads/2013/10/20185343_m.jpg

Cyanobacteria

- Single cellular organisms
- Prokaryotic
- Mostly photoautotrophic
- Live in water and moist soils
- Can be found in unicellular, colonial, and filamentous forms
- Many different species
- Truly ancient

http://www.hfmagazineonline.com/wp-content/uploads/Cyanobacteria.jpg

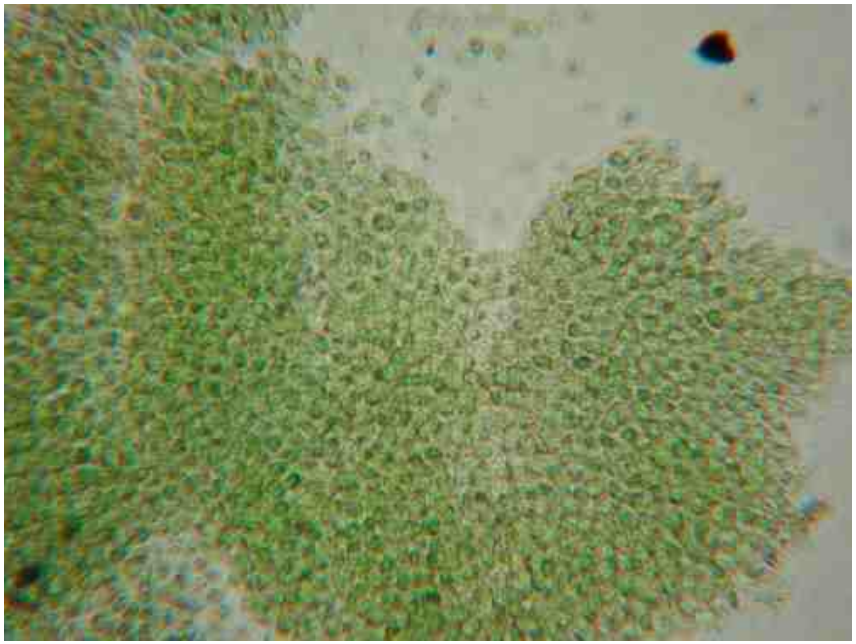
Cyanobacteria

- Characteristic color
- Bluish tinge due to phycocyanin and allophycocyanin
  - Accessory proteins to chlorophyll
- “Cyano” refers to color cyan

http://nhpr.org/post/something-wild-cyanobacteria

Microcystis sp.

- Genus of cyanobacteria
- Capable of producing microcystins
- Number of sub species
  - *M. aeruginosa* is of most concern in Iowa
    - Produces toxins



https://microbewiki.kenyon.edu/images/1/13/Microcystis_picture.jpg

Routes of Exposure

- Numerous potential exposure routes
  - Drinking water
  - Swimming
  - Boating/watersports
- Small children and small animals (such as dogs) are especially susceptible

http://www.mckenziewaterskischool.com/resources/-58.jpg

How do we test for microcystins?

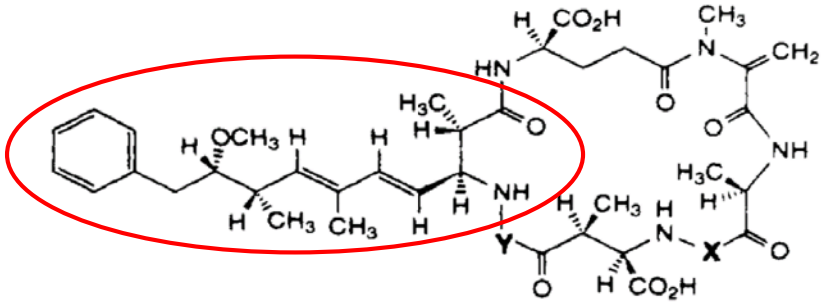
Testing For Microcystins

- Several Methods of Testing
- Screening Methods
  - Can only provide rough picture
  - Rapid
  - Inexpensive with large batch volumes
- Analytical Methods
  - Can provide detailed understanding of variants
  - Labor intensive
  - Requires expensive equipment

http://www.castlemedical.com/blog/images/17.jpg

ELISA

- Enzyme-Linked Immunosorbent Assay (ELISA)
  - Plate-based method
  - Targets specific part of microcystin molecule (ADDA)
  - Cannot discriminate between microcystin variants
  - Vulnerable to false positives



Sample Collection and Preparation

- Collect in glass or PETG containers
- Neutralize disinfectants with sodium thiosulfate
- Chill samples immediately after collection
- Freeze samples upon arrival at the laboratory

Image B

Sample Collection and Preparation

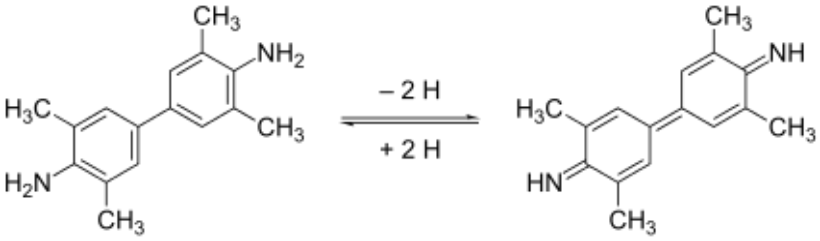
- Freeze/thaw an aliquot of the sample 3 times to lyse algal cells
- Filter samples (if turbid) through glass fiber filters
  - Other materials may cause analyte loss

Testing For Microcystins

http://www.bio-rad.com/webroot/web/images/lse/products/protein_analysis_kits/product_overlay_content/global/elisa_howkitworks.jpg

- ADDA analog bound to plate wells
- Primary antibodies added complex with analog and analyte in solution
- Secondary antibody-horseradish peroxidase (HRP) conjugates complex with primary antibodies

Testing For Microcystins



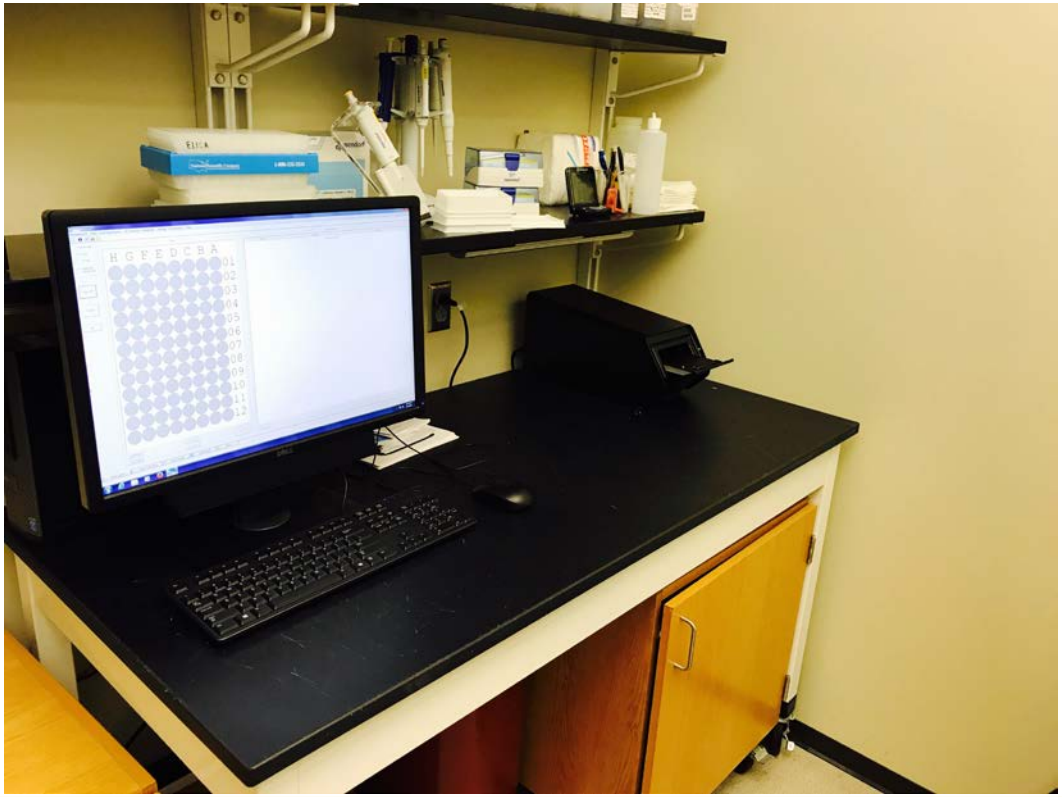
https://upload.wikimedia.org/wikipedia/commons/thumb/d/d6/3%2C3%27%2C5%2C5%27-tetramethyl--1%2C1%27-biphenyl--4%2C4%27-diamine_oxidation.svg/425px-3%2C3%27%2C5%2C5%27-tetramethyl--1%2C1%27-biphenyl--4%2C4%27-diamine_oxidation.svg.png

- Color reagent, 3,3’,5,5’-tetramethylbenzene, is oxidized by HRP
- Oxidation to the diamine is then halted and stabilized by sulfuric acid resulting a yellow color
- Inverse relationship between color intensity and concentration

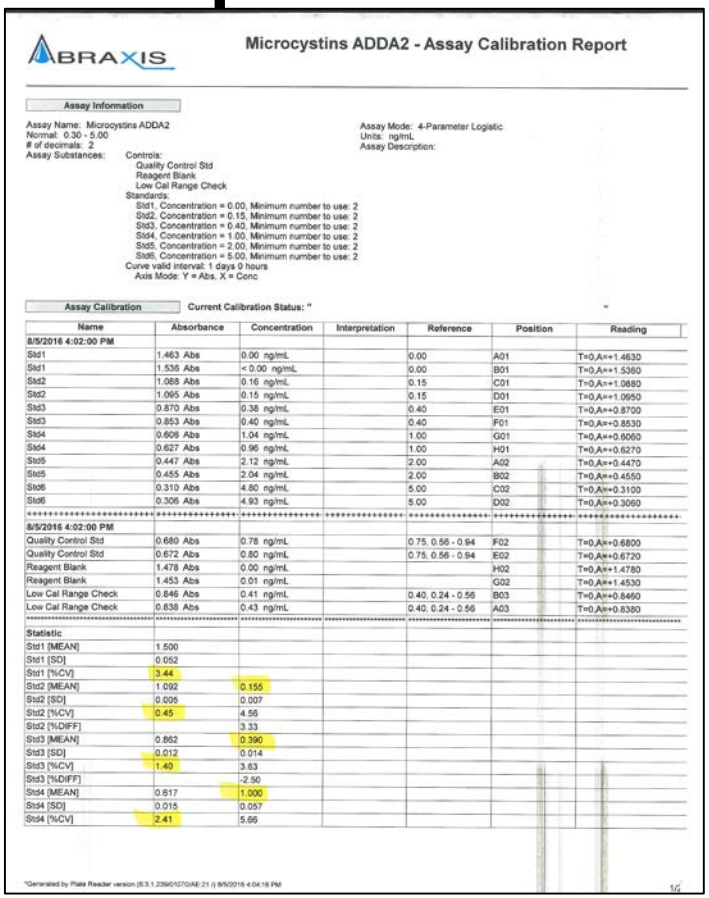
Required Equipment

- Plate reader capable of reading at 450 nm
- Computer with analytical Software
- Various micropipetters
  - Multichannel capable of 50-250 uL dispensing volume
  - Single-channel capable of dispensing 50 uL
- Plate kit
- Source of deionized water
- Glass-fiber syringe filters and syringes
- Refrigerator and Freezer

Required Equipment



Example Calibration Output



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Microcystins ADDA2 - Assay Calibration Report

Assay Information

Assay Name: Microcystins ADDA2
Normal: 0.30 - 5.00
# of decimals: 2
Assay Substances:
Controls:
Quality Control Std
Reagent Blank
Low Cal Range Check
Standards:
Std1, Concentration = 0.00, Minimum number to use: 2
Std2, Concentration = 0.15, Minimum number to use: 2
Std3, Concentration = 0.40, Minimum number to use: 2
Std4, Concentration = 1.00, Minimum number to use: 2
Std5, Concentration = 2.00, Minimum number to use: 2
Std6, Concentration = 5.00, Minimum number to use: 2
Curve valid interval: 1 days 0 hours
Axis Mode: Y = Abs, X = Conc

Assay Mode: 4-Parameter Logistic
Units: ng/mL
Assay Description:

Assay Calibration Current Calibration Status: "

Name	Absorbance	Concentration	Interpretation	Reference	Position	Reading
8/5/2016 4:02:00 PM						
Std1	1.463 Abs	0.00 ng/mL		0.00	A01	T=0,A=+1.4630
Std1	1.536 Abs	< 0.00 ng/mL		0.00	B01	T=0,A=+1.5360
Std2	1.088 Abs	0.16 ng/mL		0.15	C01	T=0,A=+1.0880
Std2	1.095 Abs	0.15 ng/mL		0.15	D01	T=0,A=+1.0950
Std3	0.870 Abs	0.38 ng/mL		0.40	E01	T=0,A=+0.8700
Std3	0.853 Abs	0.40 ng/mL		0.40	F01	T=0,A=+0.8530
Std4	0.606 Abs	1.04 ng/mL		1.00	G01	T=0,A=+0.6060
Std4	0.627 Abs	0.96 ng/mL		1.00	H01	T=0,A=+0.6270
Std5	0.447 Abs	2.12 ng/mL		2.00	A02	T=0,A=+0.4470
Std5	0.455 Abs	2.04 ng/mL		2.00	B02	T=0,A=+0.4550
Std6	0.310 Abs	4.80 ng/mL		5.00	C02	T=0,A=+0.3100
Std6	0.306 Abs	4.93 ng/mL		5.00	D02	T=0,A=+0.3060
8/5/2016 4:02:00 PM						
Quality Control Std	0.680 Abs	0.78 ng/mL		0.75, 0.56 - 0.94	F02	T=0,A=+0.6800
Quality Control Std	0.672 Abs	0.80 ng/mL		0.75, 0.56 - 0.94	E02	T=0,A=+0.6720
Reagent Blank	1.478 Abs	0.00 ng/mL			H02	T=0,A=+1.4780
Reagent Blank	1.453 Abs	0.01 ng/mL			G02	T=0,A=+1.4530
Low Cal Range Check	0.846 Abs	0.41 ng/mL		0.40, 0.24 - 0.56	B03	T=0,A=+0.8460
Low Cal Range Check	0.838 Abs	0.43 ng/mL		0.40, 0.24 - 0.56	A03	T=0,A=+0.8380
Statistic						
Std1 [MEAN]	1.500					
Std1 [SD]	0.052					
Std1 [%CV]	3.44					
Std2 [MEAN]	1.092	0.155				
Std2 [SD]	0.005	0.007				
Std2 [%CV]	0.45	4.56				
Std2 [%DIFF]		3.33				
Std3 [MEAN]	0.862	0.390				
Std3 [SD]	0.012	0.014				
Std3 [%CV]	1.40	3.63				
Std3 [%DIFF]		-2.50				
Std4 [MEAN]	0.617	1.000				
Std4 [SD]	0.015	0.057				
Std4 [%CV]	2.41	5.66				

*Generated by Plate Reader version (6.3.1.239/0107/AE:21.0) 8/5/2016 4:04:16 PM

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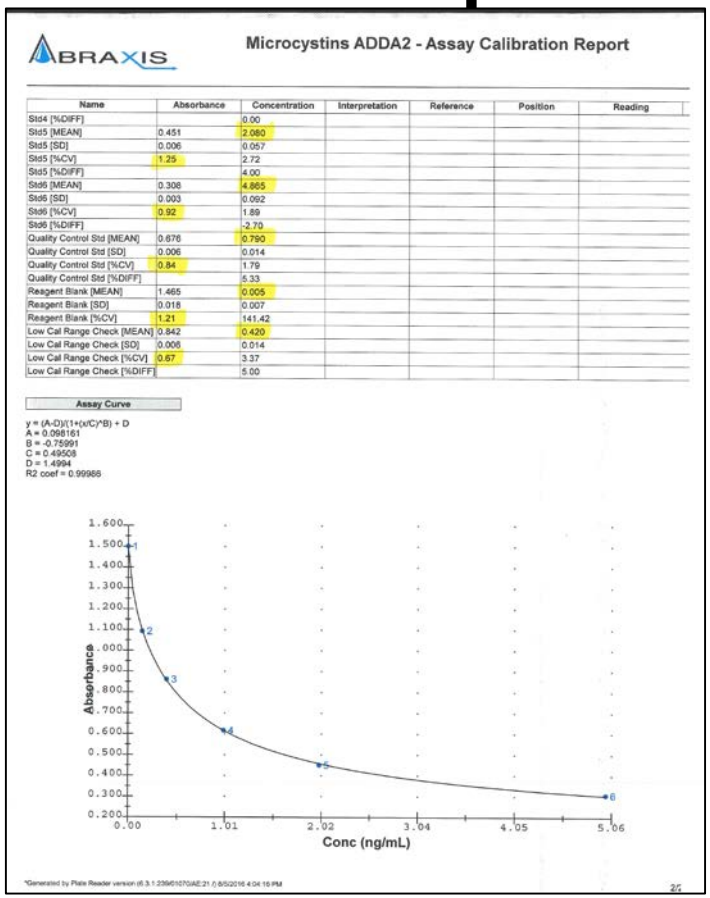

BRAXIS

Microcystins ADDA2 - Assay Calibration Report

Name	Absorbance	Concentration	Interpretation	Reference	Position	Reading
Std4 [%DIFF]		0.00				
Std5 [MEAN]	0.451	2.080				
Std5 [SD]	0.006	0.057				
Std5 [%CV]	1.25	2.72				
Std5 [%DIFF]		4.00				
Std6 [MEAN]	0.308	4.865				
Std6 [SD]	0.003	0.092				
Std6 [%CV]	0.92	1.89				
Std6 [%DIFF]		-2.70				
Quality Control Std [MEAN]	0.676	0.790				
Quality Control Std [SD]	0.006	0.014				
Quality Control Std [%CV]	0.84	1.79				
Quality Control Std [%DIFF]		5.33				
Reagent Blank [MEAN]	1.465	0.005				
Reagent Blank [SD]	0.018	0.007				
Reagent Blank [%CV]	1.21	141.42				
Low Cal Range Check [MEAN]	0.842	0.420				
Low Cal Range Check [SD]	0.006	0.014				
Low Cal Range Check [%CV]	0.67	3.37				
Low Cal Range Check [%DIFF]		5.00				

Assay Curve

y = (A-D)/(1+(x/C)^B) + D
A = 0.098161
B = -0.75991
C = 0.49508
D = 1.4994
R2 coef = 0.99988

*Generated by Plate Reader version (6.3.1.239/0107/AE:21.0) 8/5/2016 4:04:16 PM

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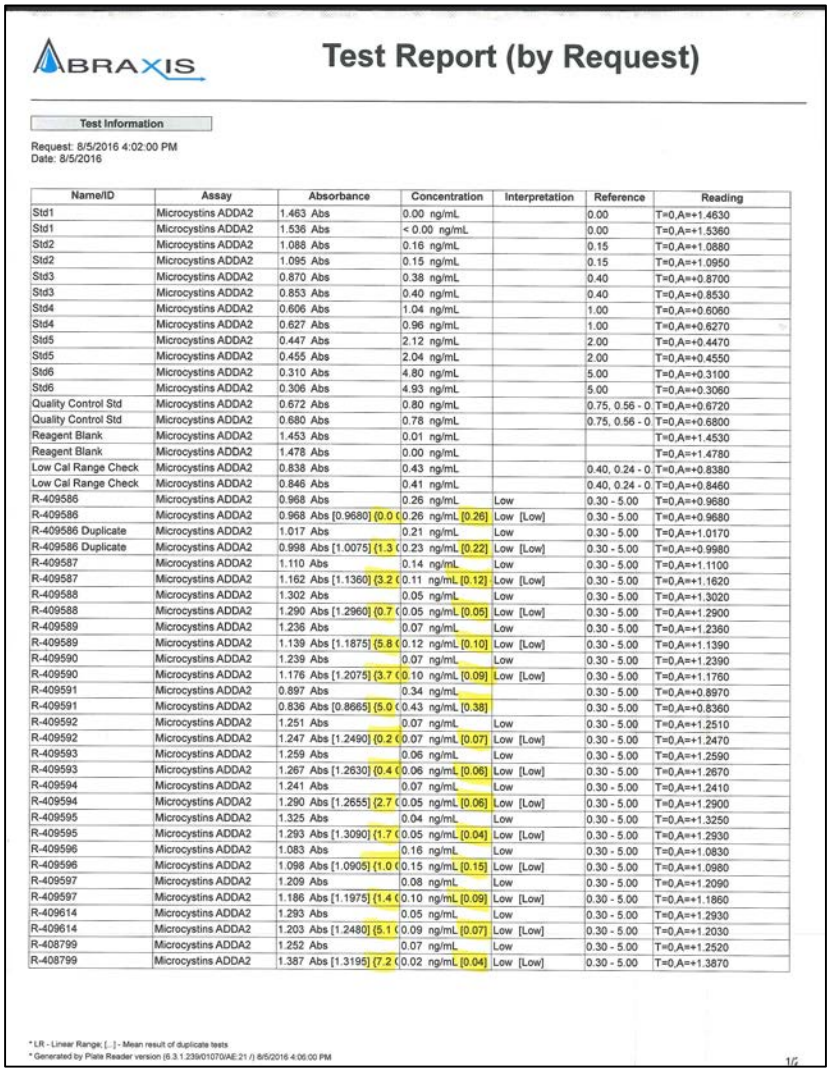
The University of Iowa

State Hygienic Laboratory — Iowa's Environmental and Public Health Laboratory — Est. 1904 — The University of Iowa



ABRAXIS

Test Report (by Request)

Test Information

Request: 8/5/2016 4:02:00 PM
Date: 8/5/2016

Name/ID	Assay	Absorbance	Concentration	Interpretation	Reference	Reading
Std1	Microcystins ADDA2	1.463 Abs	0.00 ng/mL		0.00	T=0,A=+1.4630
Std1	Microcystins ADDA2	1.536 Abs	< 0.00 ng/mL		0.00	T=0,A=+1.5360
Std2	Microcystins ADDA2	1.088 Abs	0.16 ng/mL		0.15	T=0,A=+1.0880
Std2	Microcystins ADDA2	1.095 Abs	0.15 ng/mL		0.15	T=0,A=+1.0950
Std3	Microcystins ADDA2	0.870 Abs	0.38 ng/mL		0.40	T=0,A=+0.8700
Std3	Microcystins ADDA2	0.853 Abs	0.40 ng/mL		0.40	T=0,A=+0.8530
Std4	Microcystins ADDA2	0.606 Abs	1.04 ng/mL		1.00	T=0,A=+0.6060
Std4	Microcystins ADDA2	0.627 Abs	0.96 ng/mL		1.00	T=0,A=+0.6270
Std5	Microcystins ADDA2	0.447 Abs	2.12 ng/mL		2.00	T=0,A=+0.4470
Std5	Microcystins ADDA2	0.455 Abs	2.04 ng/mL		2.00	T=0,A=+0.4550
Std6	Microcystins ADDA2	0.310 Abs	4.80 ng/mL		5.00	T=0,A=+0.3100
Std6	Microcystins ADDA2	0.306 Abs	4.93 ng/mL		5.00	T=0,A=+0.3060
Quality Control Std	Microcystins ADDA2	0.672 Abs	0.80 ng/mL		0.75, 0.56 - 0	T=0,A=+0.6720
Quality Control Std	Microcystins ADDA2	0.680 Abs	0.78 ng/mL		0.75, 0.56 - 0	T=0,A=+0.6800
Reagent Blank	Microcystins ADDA2	1.453 Abs	0.01 ng/mL			T=0,A=+1.4530
Reagent Blank	Microcystins ADDA2	1.478 Abs	0.00 ng/mL			T=0,A=+1.4780
Low Cal Range Check	Microcystins ADDA2	0.838 Abs	0.43 ng/mL		0.40, 0.24 - 0	T=0,A=+0.8380
Low Cal Range Check	Microcystins ADDA2	0.846 Abs	0.41 ng/mL		0.40, 0.24 - 0	T=0,A=+0.8460
R-409586	Microcystins ADDA2	0.968 Abs	0.26 ng/mL	Low	0.30 - 5.00	T=0,A=+0.9680
R-409586	Microcystins ADDA2	0.968 Abs [0.9680] {0.0 (	0.26 ng/mL [0.26]	Low [Low]	0.30 - 5.00	T=0,A=+0.9680
R-409586 Duplicate	Microcystins ADDA2	1.017 Abs	0.21 ng/mL	Low	0.30 - 5.00	T=0,A=+1.0170
R-409586 Duplicate	Microcystins ADDA2	0.998 Abs [1.0075] {1.3 (	0.23 ng/mL [0.22]	Low [Low]	0.30 - 5.00	T=0,A=+0.9980
R-409587	Microcystins ADDA2	1.110 Abs	0.14 ng/mL	Low	0.30 - 5.00	T=0,A=+1.1100
R-409587	Microcystins ADDA2	1.162 Abs [1.1360] {3.2 (	0.11 ng/mL [0.12]	Low [Low]	0.30 - 5.00	T=0,A=+1.1620
R-409588	Microcystins ADDA2	1.302 Abs	0.05 ng/mL	Low	0.30 - 5.00	T=0,A=+1.3020
R-409588	Microcystins ADDA2	1.290 Abs [1.2960] {0.7 (	0.05 ng/mL [0.05]	Low [Low]	0.30 - 5.00	T=0,A=+1.2900
R-409589	Microcystins ADDA2	1.236 Abs	0.07 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2360
R-409589	Microcystins ADDA2	1.139 Abs [1.1875] {5.8 (	0.12 ng/mL [0.10]	Low [Low]	0.30 - 5.00	T=0,A=+1.1390
R-409590	Microcystins ADDA2	1.239 Abs	0.07 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2390
R-409590	Microcystins ADDA2	1.176 Abs [1.2075] {3.7 (	0.10 ng/mL [0.09]	Low [Low]	0.30 - 5.00	T=0,A=+1.1760
R-409591	Microcystins ADDA2	0.897 Abs	0.34 ng/mL		0.30 - 5.00	T=0,A=+0.8970
R-409591	Microcystins ADDA2	0.836 Abs [0.8665] {5.0 (	0.43 ng/mL [0.38]		0.30 - 5.00	T=0,A=+0.8360
R-409592	Microcystins ADDA2	1.251 Abs	0.07 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2510
R-409592	Microcystins ADDA2	1.247 Abs [1.2490] {0.2 (	0.07 ng/mL [0.07]	Low [Low]	0.30 - 5.00	T=0,A=+1.2470
R-409593	Microcystins ADDA2	1.259 Abs	0.06 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2590
R-409593	Microcystins ADDA2	1.267 Abs [1.2630] {0.4 (	0.06 ng/mL [0.06]	Low [Low]	0.30 - 5.00	T=0,A=+1.2670
R-409594	Microcystins ADDA2	1.241 Abs	0.07 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2410
R-409594	Microcystins ADDA2	1.290 Abs [1.2655] {2.7 (	0.05 ng/mL [0.06]	Low [Low]	0.30 - 5.00	T=0,A=+1.2900
R-409595	Microcystins ADDA2	1.325 Abs	0.04 ng/mL	Low	0.30 - 5.00	T=0,A=+1.3250
R-409595	Microcystins ADDA2	1.293 Abs [1.3090] {1.7 (	0.05 ng/mL [0.04]	Low [Low]	0.30 - 5.00	T=0,A=+1.2930
R-409596	Microcystins ADDA2	1.083 Abs	0.16 ng/mL	Low	0.30 - 5.00	T=0,A=+1.0830
R-409596	Microcystins ADDA2	1.098 Abs [1.0905] {1.0 (	0.15 ng/mL [0.15]	Low [Low]	0.30 - 5.00	T=0,A=+1.0980
R-409597	Microcystins ADDA2	1.209 Abs	0.08 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2090
R-409597	Microcystins ADDA2	1.186 Abs [1.1975] {1.4 (	0.10 ng/mL [0.09]	Low [Low]	0.30 - 5.00	T=0,A=+1.1860
R-409614	Microcystins ADDA2	1.293 Abs	0.05 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2930
R-409614	Microcystins ADDA2	1.203 Abs [1.2480] {5.1 (	0.09 ng/mL [0.07]	Low [Low]	0.30 - 5.00	T=0,A=+1.2030
R-408799	Microcystins ADDA2	1.252 Abs	0.07 ng/mL	Low	0.30 - 5.00	T=0,A=+1.2520
R-408799	Microcystins ADDA2	1.387 Abs [1.3195] {7.2 (	0.02 ng/mL [0.04]	Low [Low]	0.30 - 5.00	T=0,A=+1.3870

* LR - Linear Range; [...] - Mean result of duplicate tests
* Generated by Plate Reader version (6.3.1.239/01070/AE:21 /) 8/5/2016 4:06:00 PM

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Example Result Output

- Results reported as average of two replicates
- Coefficient of variation given on report
- Flags provided for data interpretation

ELISA-Based Methods

EPA
United States
Environmental Protection
Agency

Method 546: Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by Adda Enzyme-Linked Immunosorbent Assay

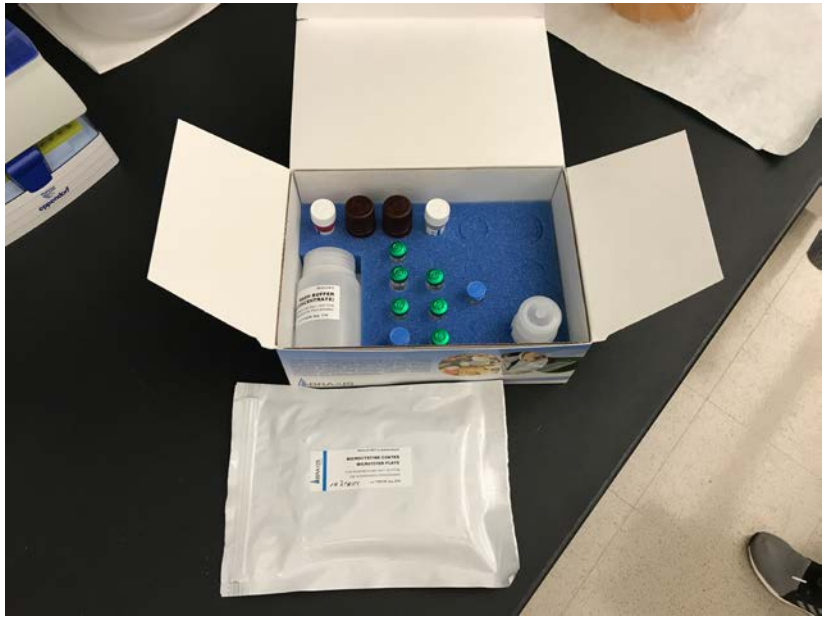
Microcystins-ADDA ELISA (Microtiter Plate)

Enzyme-Linked Immunosorbent Assay for the Congener-Independent* Determination of Microcystins and Nodularins in Water Samples

ABRAXIS

Similarities Between ELISA Methods

- Utilize ELISA plate kit
- Six point calibration curve
  - 0.0 to 5.0 ng/mL
- Second source standard
- Laboratory reagent blank
- All controls and samples analyzed in duplicate



Contrasting ELISA Methods

- Abraxis Kit Method
  - Minimal quality control
  - All materials contained within the kit
  - No procedures for demonstration of capability or detection limit determination
    - Detection limit specified by kit as 0.15 ng/mL
- EPA 546
  - UCMR4 mandated method
  - Requires extensive quality control
    - LFB, LRB, LFSM, LFSMD
  - Demonstration of capability outlined rigorously
  - Minimum reporting limit verified experimentally

Abraxis Kit Method Quality Control

- Replicates: CVs <10% for standards, 15% for samples/ controls
- Quality Control Standard: ±25%
- Laboratory Reagent Blank <Reporting Limit

H. Evaluation

The evaluation of the ELISA can be performed using commercial ELISA evaluation programs such as 4-Parameter (preferred) or Logit/Log. For a manual evaluation, calculate the mean absorbance value for each of the standards. Calculate the $\%B/B_0$ for each standard by dividing the mean absorbance value for each standard by the Zero Standard (Standard 0) mean absorbance. Construct a standard curve by plotting the $\%B/B_0$ for each standard on the vertical linear (y) axis versus the corresponding Microcystins concentration on the horizontal logarithmic (x) axis on graph paper. $\%B/B_0$ for the control and samples will then yield levels in ppb of Microcystins by interpolation using the standard curve. Results can also be determined using a spreadsheet macro available from Abraxis upon request.

The concentrations of the samples are determined using the standard curve run with each test. Samples showing a lower concentration of Microcystins than standard 1 (0.15 ppb) should be reported as containing < 0.15 ppb of Microcystins. Samples showing a higher concentration than standard 5 (5.0 ppb) must be diluted to obtain accurate results. The concentration of the positive control provided should be 0.75 ± 0.185 ppb.

Semi-quantitative results can be derived by simple comparison of the sample absorbances to the absorbances of the standards. Samples with lower absorbances than a standard will have concentrations of Microcystins greater than that standard. Samples which have higher absorbances than a standard will have concentrations of Microcystins less than that standard.

Performance Data

Test sensitivity:	The detection limit for this assay, based on MC-LR, is 0.10 ppb (µg/L).
Test reproducibility:	Coefficients of variation (CVs) for standards: <10%; for samples: <15%.
Selectivity*:	The assay exhibits very good cross-reactivity with all cyanobacterial cyclic peptide toxin congeners tested to date (see cross-reactivity illustration below).

EPA 546 Quality Control

Table 11. Analysis Batch QC Requirements

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
10	ELISA Calibration	Use kit-recommended levels and concentrations. Two well replicates (Sect. 3.1.1) per standard.	%CV of absorbance ≤10%; ≤15% allowed for 1 pair. $r^2 \geq 0.98$.
9.2.1	Well replicates	Assay field and QC samples in two wells.	Sample invalid if %CV of absorbance values >15%
9.2.2	Laboratory Reagent Blank (LRB)	Lyse one LRB per Analysis Batch. Assay in duplicate on opposite sides of the plate.	MC/NOD concentration must be less than one-half the Minimum Reporting Level (MRL) in each LRB.
9.2.3	Low Calibration Verification (Low-CV)	Calibration standard at, or below, the MRL concentration. One per Analysis Batch.	Percent recovery ≥50% and ≤150% of the true value
9.2.4	Laboratory Fortified Blank (LFB)	Reagent water fortified near the EC_{50}. Lyse and assay 2 per Analysis Batch.	Percent recovery for each LFB ≥60% and ≤140% of the true value
9.2.5	Laboratory Fortified Sample Matrix (LFSM) and LFSM Duplicate	Fortify near the EC_{50} and twice native concentration. One set in Analysis Batches containing drinking water; two if 20 or more field samples. One set in Analysis Batches containing ambient water; two if 20 or more field samples.	Mean percent recovery of LFSM and LFSMD pair ≥60% and ≤140%. Relative percent difference (RPD) ≤40%. Qualify results for samples failing these limits as "suspect–matrix".
9.2.6	Quality Control Sample (QCS)	Assay 1 QCS for each new lot of calibration standards. Prepare the QCS near the EC_{50} with MC-LR from a source independent of the calibration standards.	Percent recovery ≥70% and ≤130% of the true value

- QC similar to Abraxis kit method but much more extensive
- Requires a stock standard of microcystin-LR for spiking

EPA 546 Demonstration of Capability

EPA 546 Method Validation										Date	2017-03-29	Analyst	mscaffin	
Replicate	MRL (µg/L)	Replicate	LFB (µg/L)	Replicate	LRB (µg/L)	Replicate	LFSM (µg/L)	LFSMD (µg/L)	Tap Water (µg/L)	Mean % Recovery	RPD	QCS (µg/L)	% Recovery	Low-CV (µg/L)	% Recovery
1	0.28	1	0.41	1	0.02	1	0.46	0.41	0.01	84%	11%	0.94	125%	0.11	73%
2	0.33	3	0.46	3	0.02	2	0.44	0.47	0.01	88%	8%				
3	0.29	4	0.46	4	0.02										
4	0.29	5	0.45	5	0.01										
5	0.27	6	0.45	2	0.01										
6	0.31	7	0.44												
7	0.31	2	0.43												
Mean	0.30	Mean	0.44												
Mean % Recovery	99%	Mean % Recovery	88%												
Stdev	0.021	Stdev	0.02												
RSD	2.1%	RSD	4%												
HR(PIR)	0.08														
Upper PIR Limit	127%														
Lower PIR Limit	72%														

Table 10. Initial Demonstration of Capability (IDC) QC Requirements

Method Reference	Requirement	Specification	Acceptance Criteria
9.1.1	Demonstration of precision and accuracy	Lyse and assay 7 replicate Laboratory Fortified Blanks (LFBs) at 0.50 µg/L.	Percent relative standard deviation ≤15%. Mean percent recovery ≥70% and ≤130%.
9.1.2	Demonstration of acceptable system background	Lyse and assay 5 Laboratory Reagent Blanks (LRBs) distributed throughout a plate.	MC concentration must be less than one-half the Minimum Reporting Level (MRL) in each LRB.
9.1.3	MRL confirmation	Fortify and analyze 7 replicate LFBs at the proposed MRL concentration. Confirm that the Upper Prediction Interval of Results (PIR) and Lower PIR meet the recovery criteria.	Upper PIR ≤150% Lower PIR ≥50%
9.1.4	Quality Control Sample (QCS)	Prepare a QCS near the EC_{50} with MC-LR from a source independent from the calibration standards.	Percent recovery ≥70% and ≤130% of the true value

ELISA-Based Methods

Pros

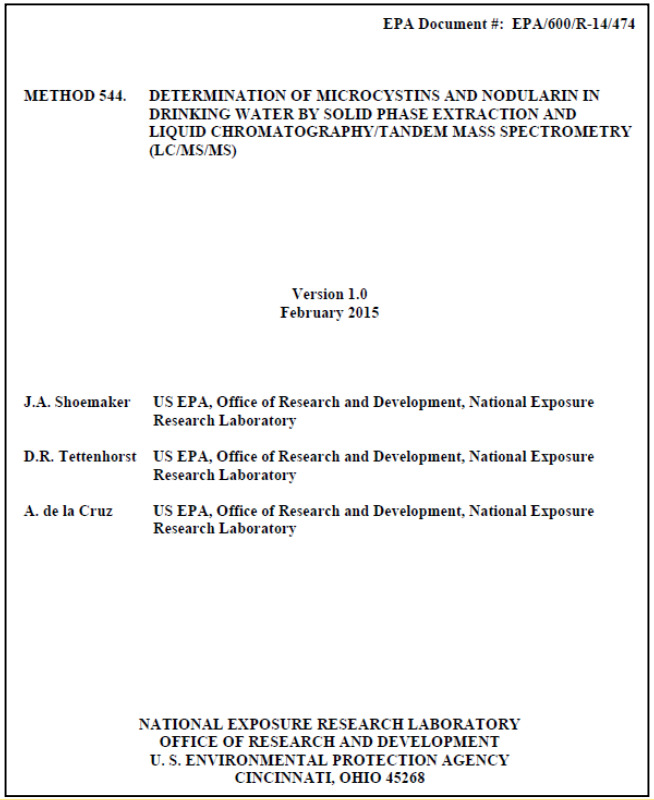
- Does not require expensive equipment
- Quick turnaround
  - Receipt to results in 4-6 hours
- Low susceptibility to false negatives
- Inexpensive with large numbers of samples
- Proven technology

Cons

- Highly technique dependent
- Very susceptible to matrix interferences/ false positives
- Cannot identify variants
- Kits are very expensive
- Must establish new calibration curve with each run
- Semi-quantitative at best
- EPA 546 quality control can be onerous for a screening test

Analytical Method

- EPA 544
- LC-MS/MS based method
- Validated for a number of microcystin variants (MC-LA, LF, LR, LY, RR, YR) and nodularin-R



EPA Document #: EPA/600/R-14/474

METHOD 544. DETERMINATION OF MICROCYSTINS AND NODULARIN IN DRINKING WATER BY SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

Version 1.0
February 2015

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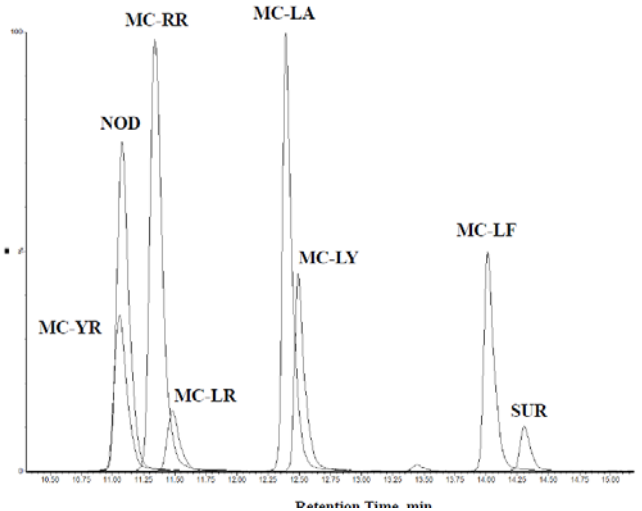
EPA 544

- Spike 500 mL sample with deuterated MC-LR surrogate
- Filter sample
- Lyse filtered solids via freezing and combine washes with filtrate
- Extract analytes of interest using SPE cartridge
- Elute with methanol, concentrate to 1 mL

FIGURE 2. EXAMPLE CHROMATOGRAM (OVERLAID MS/MS SEGMENTS) OF A CALIBRATION STANDARD WITH METHOD 544 ANALYTES AT CONCENTRATION LEVELS OF 187.5-1000 ng/L.



EPA 544

- Separate analytes utilizing LC-MS/MS
- Tandem mass spectrometry allows for the separation, identification, and quantitation of co-eluting analytes

TABLE 4. MS/MS METHOD CONDITIONS[a]

Segment[b]	Analyte	Precursor Ion[c] (*m/z*)		Product Ion[c,d] (*m/z*)	Cone Voltage (v)	Collision Energy[e] (v)
1	MC-YR	523.4	$[M+2H]^{2+}$	134.9	20	15
1	Nodularin-R	825.4	$[M+H]^{+}$	134.9	45	55
1	MC-RR	519.9	$[M+2H]^{2+}$	134.9	35	30
1	MC-LR	995.5	$[M+H]^{+}$	134.9	60	65
2	MC-LA	910.5	$[M+H]^{+}$	776.4	40	20
2	MC-LY	1002.5	$[M+H]^{+}$	134.9	40	60
3	MC-LF	986.5	$[M+H]^{+}$	134.9	40	60
3	C_2D_5-MC-LR (SUR)	1028.6	$[M+H]^{+}$	134.9	55	60

[a] An LC/MS/MS chromatogram of the analytes is shown in Figure 2.

[b] Segments are time durations in which single or multiple scan events occur.

[c] During MS and MS/MS optimization, the analyst should determine the precursor and product ion masses to one decimal place by locating the apex of the mass spectral peak place (e.g., *m/z* 523.4→134.9 for MC-YR). These precursor and product ion masses (with one decimal place) should be used in the MS/MS method for all analyses.

[d] Ions used for quantitation purposes.

[e] Argon used as collision gas at a flow rate of 0.3 mL/min.

EPA 544

- Extensive quality control and demonstration of capability requirements

TABLE 12. ONGOING QUALITY CONTROL REQUIREMENTS (SUMMARY)

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
Sect. 8.4	Sample Holding Time	28 days with appropriate preservation and storage as described in Sections 8.1-8.4.	Sample results are valid only if samples are extracted within the sample holding time.
Sect. 8.4	Extract Holding Time	28 days when stored at ≤ -4 °C.	Extract results are valid only if extracts are analyzed within the extract holding time.
Sect. 9.3.1	Laboratory Reagent Blank (LRB)	One LRB with each extraction batch of up to 20 field samples.	Demonstrate that all method analytes are below 1/3 the MRL, and confirm that possible interferences do not prevent quantification of method analytes. If targets exceed 1/3 the MRL or if interferences are present, results for these subject analytes in the extraction batch are invalid.
Sect. 9.3.3	Laboratory Fortified Blank (LFB)	One LFB is required for each extraction batch of up to 20 field samples. Rotate the fortified concentrations between low, medium, and high amounts.	Results of LFB analyses must be 70-130% of the true value for each method analyte for all fortified concentrations except the lowest CAL point. Results of the LFBs corresponding to the lowest CAL point for each method analyte must be 50-150% of the true value.
Sect. 9.3.4	Surrogate Standards (SUR)	The surrogate is added to all CAL standards and samples, including QC samples. Calculate SUR recoveries.	SUR recovery in extracts must be 60-130% of the true value. SUR recovery in CCCs must be 70-130% of the true value. If a SUR fails these criteria, report all results for sample as suspect/SUR recovery.
Sect. 9.3.5	Laboratory Fortified Sample Matrix (LFSM)	Analyze one LFSM per extraction batch (20 samples or less) fortified with method analytes at a concentration greater than or equal to the native concentration, if known. Calculate LFSM recoveries.	Recoveries at mid and high levels should be within 60-140% and within 50-150% at the low-level fortified amount (near the MRL). If these criteria are not met, results are labeled suspect due to matrix effects.
Sect. 9.3.6	Field Duplicates (FD) or Laboratory Fortified Sample Matrix Duplicate (LFSMD)	Extract and analyze at least one FD or LFSMD with each extraction batch (20 samples or less). A LFSMD may be substituted for a FD when the frequency of detects are low. Calculate RPDs.	See Sect. 9.3.5 and 9.3.6 for instructions on the interpretation of LFSM and FD results.
Sect. 9.3.7	Quality Control Sample (QCS)	Analyze at least quarterly or when preparing new standards, as well as during the IDC.	Results should be within 70-130% of true value.

TABLE 11. INITIAL DEMONSTRATION OF CAPABILITY QUALITY CONTROL REQUIREMENTS

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
Sect. 9.2.1 and 9.3.1	Initial Demonstration of Low System Background	Analyze LRB prior to any other IDC steps.	Demonstrate that all method analytes are below 1/3 the MRL and that possible interferences from extraction media do not prevent the identification and quantification of method analytes.
Sect. 9.2.2	Initial Demonstration of Precision (IDP)	Analyze four to seven replicate LFBs fortified near the midrange calibration concentration.	%RSD must be <30%
Sect. 9.2.3	Initial Demonstration of Accuracy (IDA)	Calculate average recovery for replicates used in IDP.	Mean recovery ± 30% of true value
Sect. 9.2.4	Minimum Reporting Limit (MRL) Confirmation	Fortify, extract and analyze seven replicate LFBs at the proposed MRL concentration. Calculate the Mean and the Half Range (HR). Confirm that the upper and lower limits for the Prediction Interval of Result (Upper PIR, and Lower PIR, Sect. 9.2.4.2) meet the recovery criteria.	Upper PIR ≤ 150% Lower PIR ≥ 50%
Sect. 9.2.5 and 9.3.7	Quality Control Sample (QCS)	Analyze a standard from a second source, as part of IDC.	Results should be within 70-130% of true value.

NOTE: Table 11 is intended as an abbreviated summary of QC requirements provided as a convenience to the method user. Because the information has been abbreviated to fit the table format, there may be issues that need additional clarification, or areas where important additional information from the method text is needed. In all cases, the full text of the QC in Section 9 supersedes any missing or conflicting information in this table.

TABLE 12. (Continued)

Method Reference	Requirement	Specification and Frequency	Acceptance Criteria
Sect. 10.2	Initial Calibration	Use external calibration technique to generate a linear or quadratic calibration curve for each analyte. Use at least five standard concentrations. Check the calibration curve as described in Sect. 10.2.7.	When each CAL standard is calculated as an unknown using the calibration curve, the analyte results must be 70-130% of the true value for all except CAL standards ≤ MRL, which must be 50-150% of the true value. If this criterion is not met reanalyze the CAL standards, restrict the range of calibration, or select an alternate method of calibration.
Sect. 9.3.2 and Sect. 10.3	Continuing Calibration Check (CCC)	Verify initial calibration by analyzing a low level (at the MRL or below) CCC prior to analyzing samples. CCCs are then injected after every 10 field samples and after the last sample, rotating concentrations to cover the calibrated range of the instrument.	Recovery for each SUR must be within 70-130% of the true value in all CCCs. Each analyte fortified at a level ≤ MRL must calculate to be within ± 50% of the true value. The calculated concentration of the method analytes in CCCs fortified at all other levels must be within ± 30%.

NOTE: Table 12 is intended as an abbreviated summary of QC requirements provided as a convenience to the method user. Because the information has been abbreviated to fit the table format, there may be issues that need additional clarification, or areas where important additional information from the method text is needed. In all cases, the full text of the QC in Section 8-10 supersedes any missing or conflicting information in this table.

EPA 544

Pros

- Specifically identifies variants
- Extremely accurate
- Less susceptible to interferences
- Highly quantitative

Cons

- Extremely labor intensive
  - Extended sample preparation/extraction
- Requires expensive standards/surrogates
- Expensive instrumentation

Testing for Microcystins

- Conclusions
  - ELISA screening provides a fast low cost option for estimate microcystin concentrations
  - Most labs could implement this testing with minimal investment
  - ELISA possesses serious issues with regards to accuracy and necessitates large sample volumes to be profitable
  - EPA544 is an extremely accurate and detailed analysis
  - Unfortunately, EPA 544 is expensive and labor intensive
    - This is why ELISA screens was implemented in UCMR4 to reduce the volume of testing performed by this method
  - SHL has developed an LC-MS/MS method that should provide significant cost savings and improved turnaround time versus EPA 544

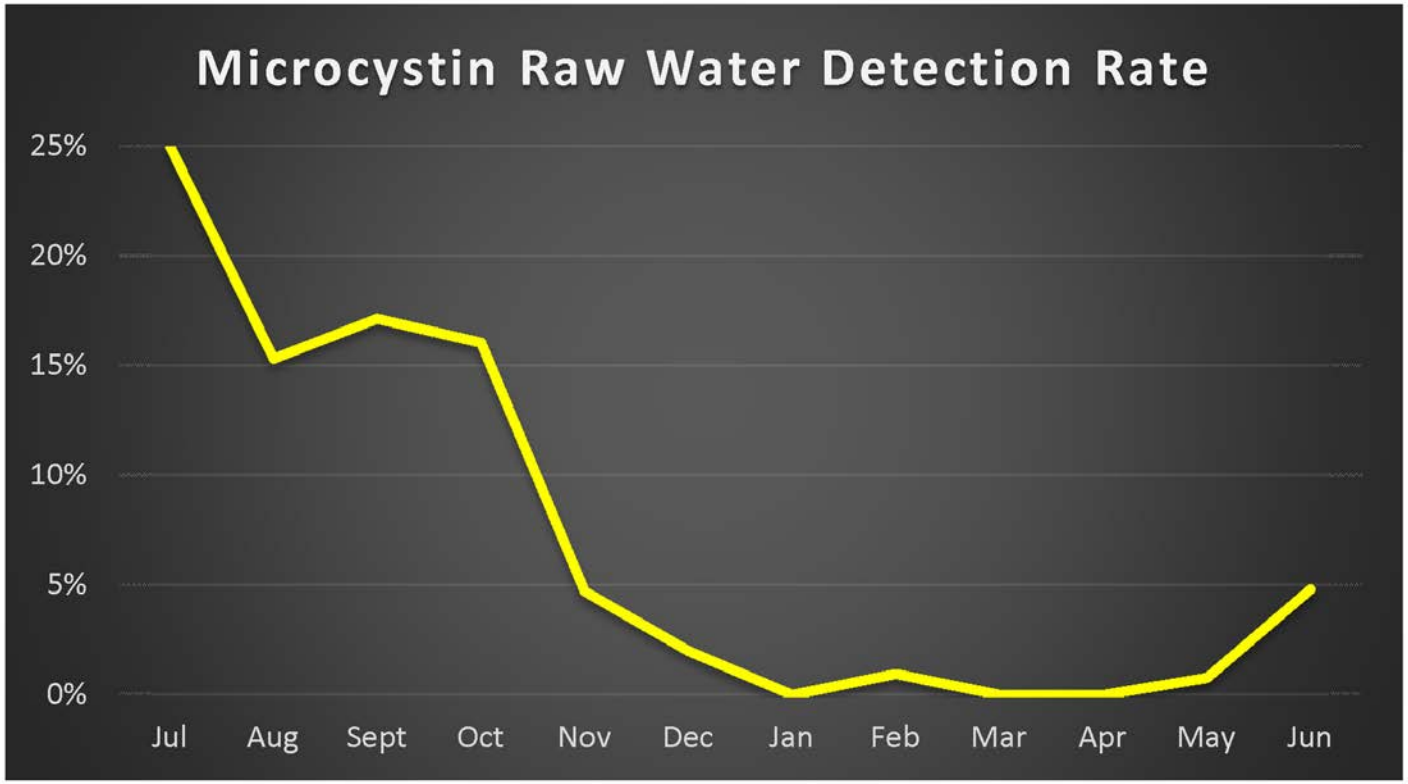
FY2017 IDNR Microcystins Project

- Goal: Evaluate prevalence of microcystins in vulnerable Iowa water supplies
- Collaboration between operators, IDNR, and SHL
- 26 water supplies participated
- Detection in raw water triggers monitoring in finished water

FY2017 IDNR Microcystins Project

- Statistics
  - 28 water sources tested
  - 854 round-trip shipments
  - 1494 analyses performed
  - 1454 individual sample collected and analyzed
    - 1365 raw water samples
    - 89 finished water samples
  - Sample collected every week for a year
- Results Overview
  - Microcystins detected in 7.1% raw water samples analyzed
  - Microcystins detected in one finished water sample
    - Extremely low, may have been a false positive
  - Seasonal and water source patterns observed

FY2017 IDNR Microcystins Project



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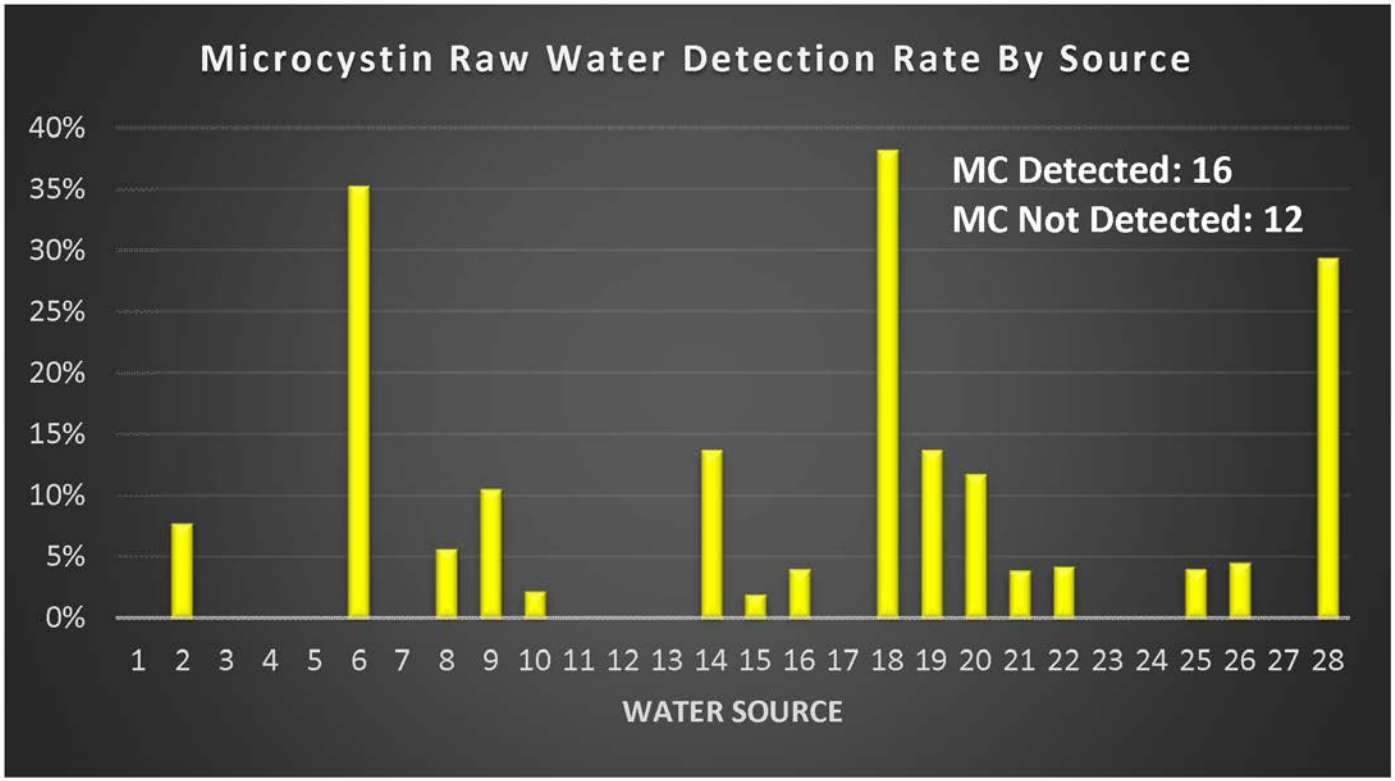

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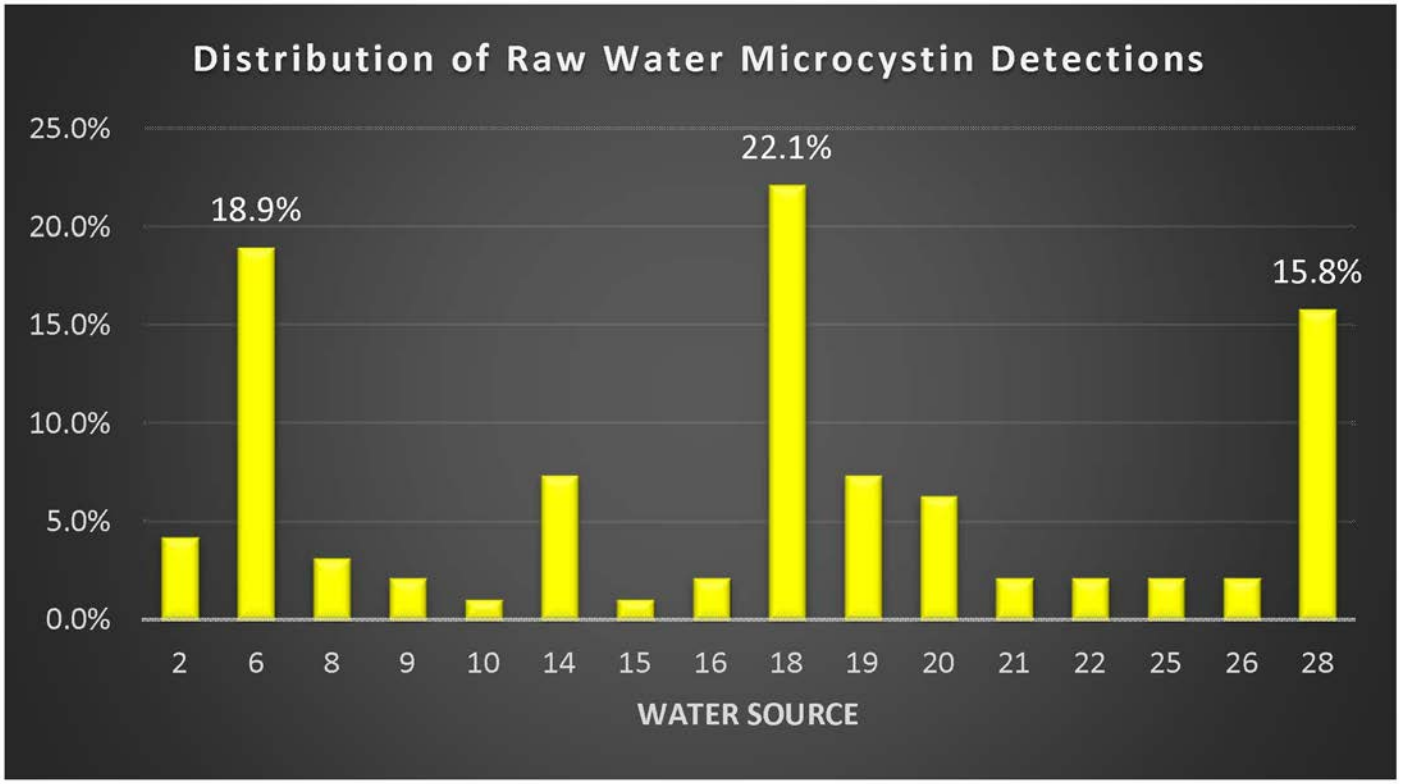

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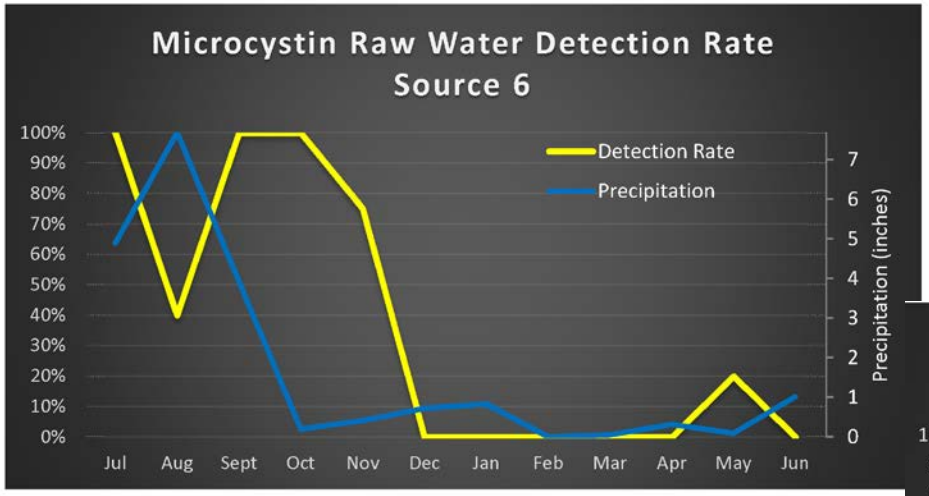

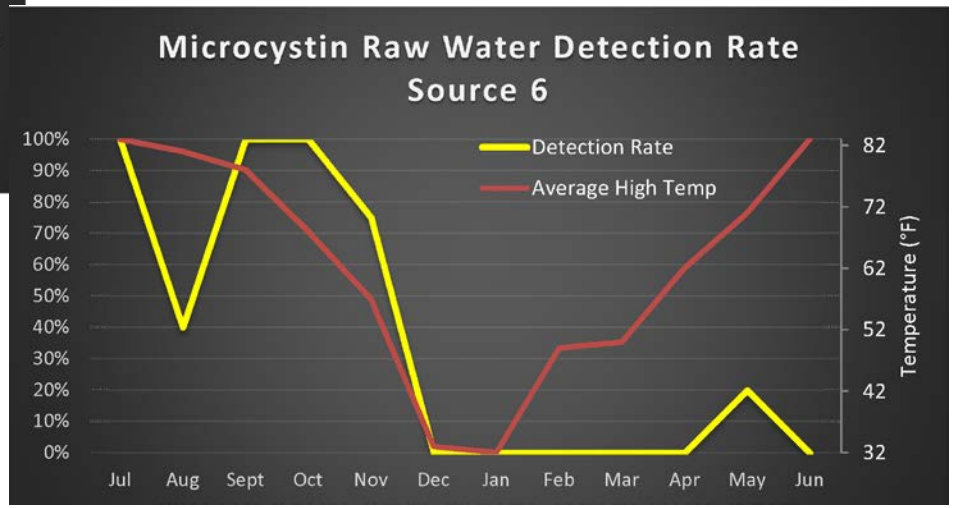

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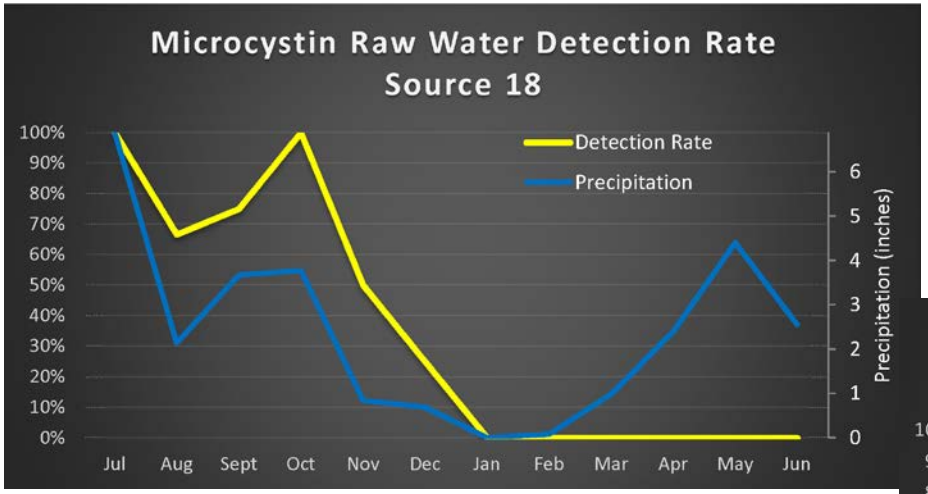

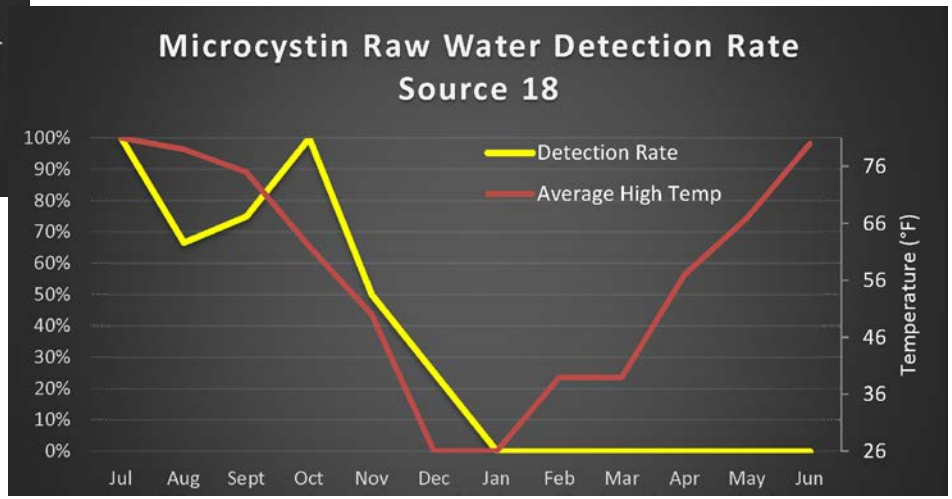

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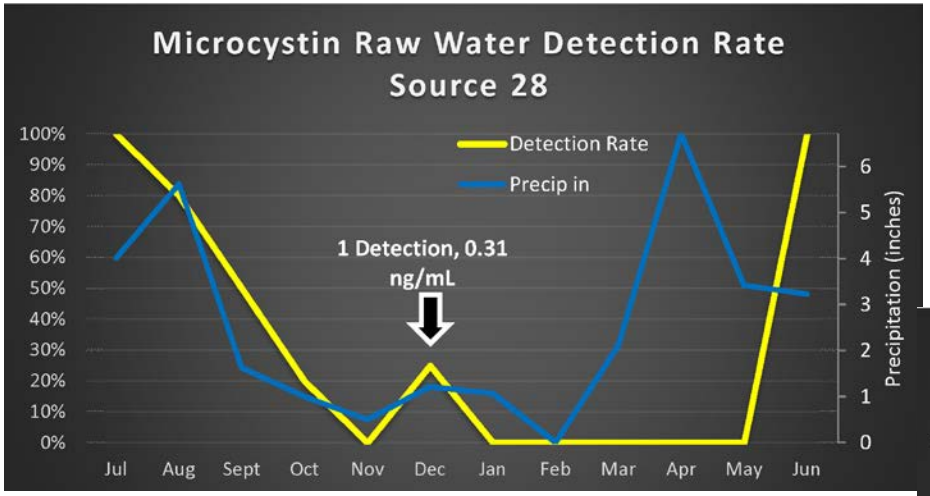

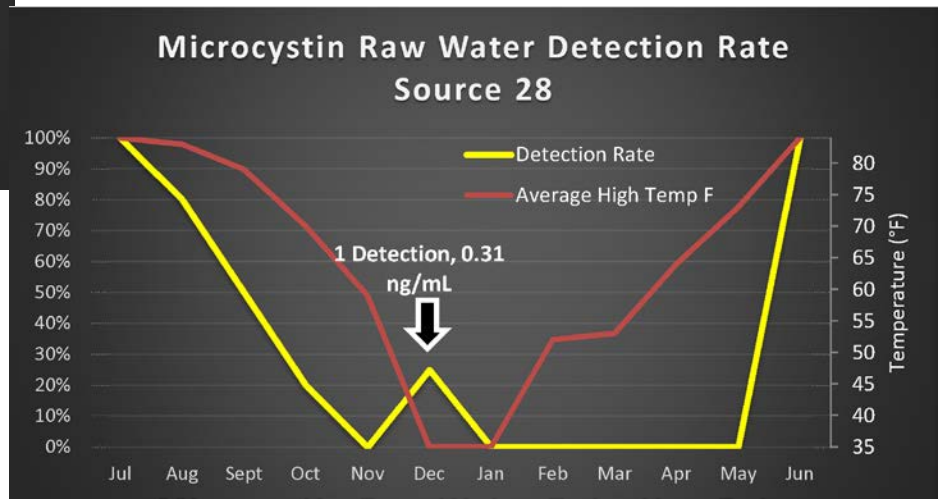

FY2017 IDNR Microcystins Project

- Conclusions
  - Follow distinct seasonal patterns
  - Appear to have strong correlations with precipitation and temperature
  - Remain an issue in water until mid-autumn
  - Persistent concentrations appear isolated to a few locations
  - Widespread transient issue

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