

EXPANDED ANALYSIS OF CYANOBACTERIAL TOXINS IN RECREATIONAL AND DRINKING WATER USING UPLC/MS/MS DETECTION...MORE TOXINS!

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Introduction

- **Microcystins** are cyclic peptides produced by cyanobacteria
- They are **produced by overgrowth of algae**, especially at higher water temperatures – **Climate change, invasive species, global trade and agricultural practices can exacerbate** the problem
- Microcystins can be **toxic for animals including humans**
- Once ingested, microcystins travel to the liver, via the bile acid transport system, where most is stored. Some remains in the blood stream and may contaminate tissue. Microcystins bind covalently to protein phosphatases thus disrupting cellular control processes. Their hepatotoxicity **may cause serious damage to the liver.**
- Over 100+ toxic variants are known



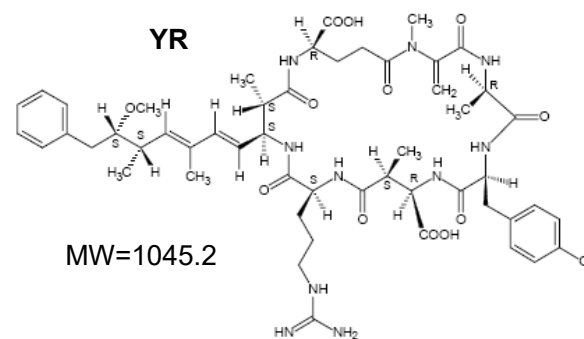
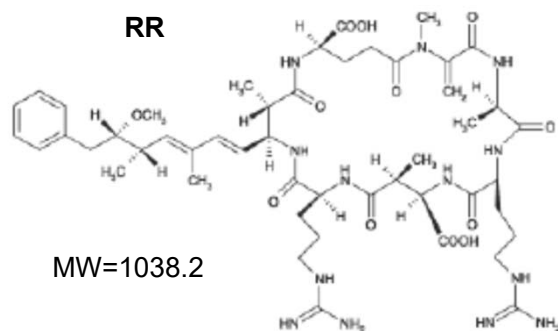
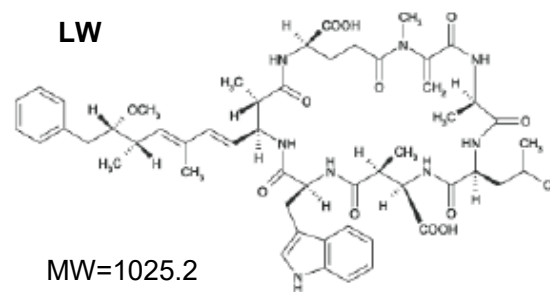
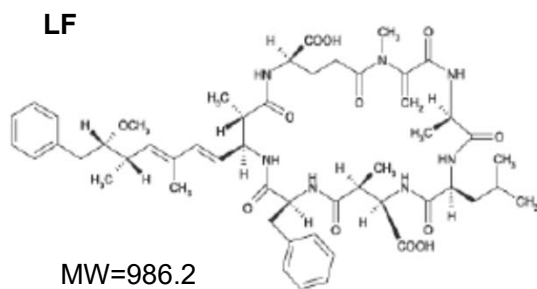
Introduction

- Microcystin-containing 'blooms' are a problem in countries worldwide including China, Brazil, Australia, the USA and much of Europe
- WHO action limit = **1000 ng/L (1ppb)** and methods are proposed including US EPA Methods 544 and 545 and European ISO 20179:2005(E) guideline
- For this reason sensitive detection is needed

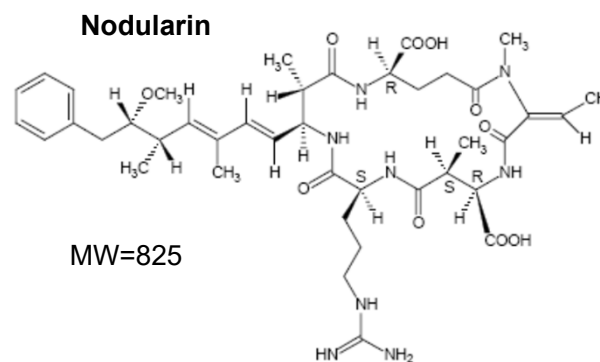
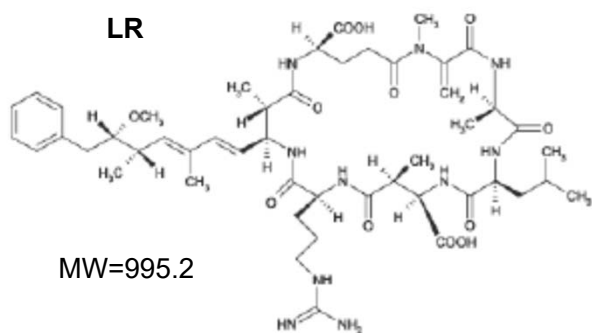


Various Microcystins

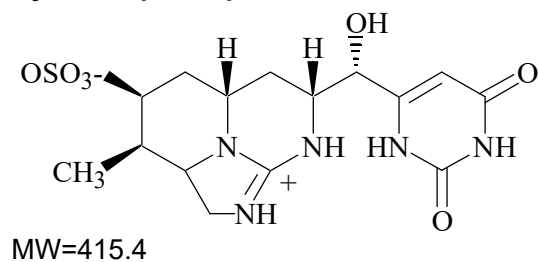
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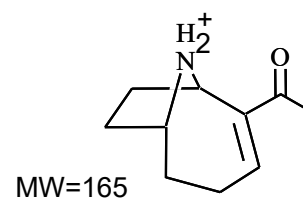
Various Microcystins and others



Cylindrospermopsin



Anatoxin



Pathway/Route of Exposure

- Recreational waters - dermal, inhalation, and ingestion
- Drinking water - ingestion, dermal, ingestion.
- Dietary Supplements – ingestion
- Vegetables and Fruits - ingestion

This is a "Global Challenge"



Microcystin Detection Assay

- Enzyme-linked Immunosorbent Assay (ELISA)
 - Uses polyclonal antibodies against different microcystin variants.
 - Samples are read spectrophotometrically to determine microcystin concentration.
 - Detection limit is low ppb
 - Cloudy or Murky samples can pose a challenge
 - Measures total toxins (not specific ones)

- High-Performance LC
 - Powerful separation capability
 - UV detection (not sensitive w/o SPE)

- LC and Mass Spectrometry
 - Offers specificity and sensitivity

EPA Guidelines for Drinking Water

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Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electro spray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS)

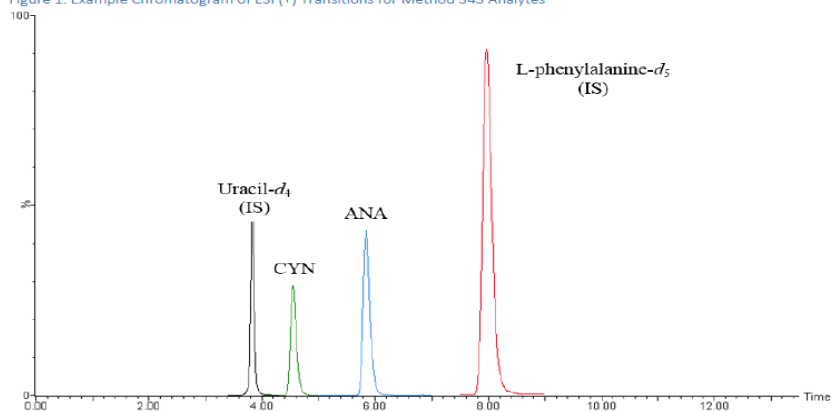
METHOD 544. DETERMINATION OF MICROCYSTINS AND NODULARIN IN DRINKING WATER BY SOLID PHASE EXTRACTION AND LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

Analyte

microcystin-LA (MC-LA)
microcystin-LF (MC-LF)
microcystin-LR (MC-LR)
microcystin-LY (MC-LY)
microcystin-RR (MC-RR)
microcystin-YR (MC-YR)
nodularin-R (NOD)

EPA separations

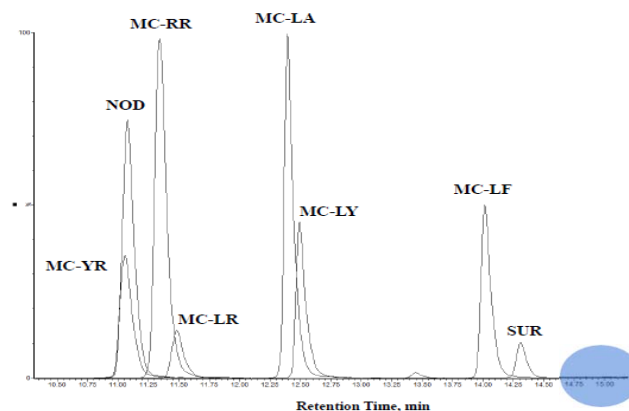
Figure 1. Example Chromatogram of ESI (+) Transitions for Method 545 Analytes



^a Waters Acquity LC system

^a Micromass Quattro Premier XE triple quadrupole mass spectrometer

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.



i 15.00

6.9.1 SPE CARTRIDGES – Waters Oasis HLB, 150 mg, 6cc divinylbenzene N-vinylpyrrolidone copolymer (Waters # 186003365).

Guidelines-WHO

What are the health-based standards or guidelines for cyanobacteria/cyanotoxins in drinking water?

(source: <http://www2.epa.gov/nutrient-policy-data/guidelines-and-recommendations#what1>)

In 1998, The World Health Organization (WHO) released a provisional drinking water guideline for microcystin-LR, excluding other known cyanotoxins since there was insufficient data to derive guideline values for these toxins. The WHO guidance value for drinking water is:

Cyanotoxin	Drinking Water Provisional Guideline
Microcystin-LR	1 µg/L

For recreational waters, the WHO concludes that a single guideline value for cyanobacteria or cyanotoxins is not appropriate. Due to the variety of possible exposures through recreational activities (contact, ingestion and inhalation) it is necessary to differentiate between the chiefly irritative symptoms caused by unknown cyanobacterial substances and the more severe health effects due to exposure to high concentrations of known cyanotoxins, particularly microcystins. The WHO guidance values for the relative probability of acute health effects during recreational exposure to cyanobacteria and microcystins are:

Relative Probability of Acute Health Effects	Cyanobacteria (cells/mL)	Microcystin-LR (µg/L)	Chlorophyll-a (µg/L)
Low	< 20,000	<10	<10
Moderate	20,000-100,000	10-20	10-50
High	100,000-10,000,000	20-2,000	50-5,000
Very High	> 10,000,000	>2,000	>5,000

Drinking Water Guidelines for Cyanotoxins



- No federal regulations for cyanobacteria/cyanotoxins in drinking water in the U.S.

Authority/Country/State	Microcystin	CYL	Anatoxin-a	Saxitoxin
World Health Organization (WHO), 2003	1 µg/L MC-LR	-	-	-
Health Canada, 2002	1.5 µg/L MC-LR	-	-	-
Brazil, 2005	1 µg/L MC-LR	15 µg/L	-	3 µg/L
Australia, 2009	1.3 µg/L MC-LR TE	1 µg/L	3 µg/L	3 µg/L
New Zealand, 2009	1 µg/L MC-LR TE	1 µg/L	6 µg/L -	-3 µg/L
Denmark	1 µg/L	-	6 µg/L	-
Singapore, Poland, Norway, China, Netherlands, Korea, Japan, Italy, France, Germany, Finland, Czech Republic	1 µg/L MC-LR	-	-	-
Ohio, 2015	0.3 µg/L bottle-fed infants and pre-school age children 1.6 µg/L school-age children and adults	0.7 µg/L bottle-fed infants and pre-school age children 3 µg/L school-age children and adults	20 µg/L	0.2 µg/L
Oregon	1 µg/L MCs	1 µg/L	3 µg/L	3 µg/L
Minnesota	0.04 µg/L MC-LR	-	-	-

In 2015, EPA developed Health Advisories (HA) for the following cyanobacterial toxins:

- [US EPA Drinking Water Health Advisory for the Cyanobacterial Toxin Cylindrospermopsin](#)
- [US EPA Drinking Water Health Advisory for the Cyanobacterial Microcystins Toxins](#)

These HAs are not regulations and should not be construed as legally enforceable federal standards. HAs may change as new information becomes available.

Cyanotoxin	Drinking Water Health Advisory (10-day)	
	Bottle-fed infants and pre-school children	School-age children and adults
Microcystins	0.3 µg/L	1.6 µg/L
Cylindrospermopsin	0.7 µg/L	3 µg/L

State Guidelines—Recreational Water

For recreational waters, the WHO concludes that a single guideline value for cyanobacteria or cyanotoxins is not appropriate. Due to the variety of possible exposures through recreational activities (contact, ingestion and inhalation) it is necessary to differentiate between the chiefly irritative symptoms caused by unknown cyanobacterial substances and the more severe health effects due to exposure to high concentrations of known cyanotoxins, particularly microcystins. The WHO guidance values for the relative probability of acute health effects during recreational exposure to cyanobacteria and the probability of microcystins concentrations are:

Relative Probability of Acute Health Effects	Cyanobacteria (cells/mL)	Microcystin-LR (µg/L)	Chlorophyll-a (µg/L)
Low	< 20,000	<10	<10
Moderate	20,000-100,000	10-20	10-50
High	100,000-10,000,000	20-2,000	50-5,000
Very High	> 10,000,000	>2,000	>5,000

Many U.S. states have implemented HAB response guidelines in the event of a significant bloom in recreational waterways. These include specific criteria for analyzing the severity of a bloom and the actions—public advisories, posted warnings, waterway or beach closures, among others—that correspond to a bloom that meets a certain threshold. For a summary of the U.S. states with guidance values, see the table below.

<https://www.epa.gov/nutrient-policy-data/guidelines-and-recommendations#what3>

State Guidelines—Recreational Water

(just a few shown)

State	Recreational Water Guidance/Action Level	Recommended Action
California	Caution Trigger Level = MCs: 0.8 µg/L; Anatoxin-a: Detect; Cylindrospermopsin: 1 µg/L Warning Tier I = MCs: 6 µg/L; Anatoxin-a: 20 µg/L; Cylindrospermopsin: 4 µg/L Danger Tier II = MCs: 20 µg/L; Anatoxin-a: 90 µg/L; Cylindrospermopsin: 17 µg/L	The trigger level of 0.8 µg/L microcystin prompts increased monitoring and the placement of a caution sign stating that people should stay away from scum and pets and livestock should be kept away from the water and scum. Microcystins refers to the sum of all measured microcystin variants. Must use an analytical method that detects <1 µg/L anatoxin-a.
Connecticut	-Visual Rank Category 1: Visible Material is not likely cyanobacteria or water is generally clear. -Visual Rank Category 2: Cyanobacteria present in low numbers. There are visible small accumulations but water is generally clear. -Visual Rank Category 3: Cyanobacteria present in high numbers. Scums may or may not be present. Water is discolored throughout. Large areas affected. Color assists to rule out sediment and other algae.	-Visual Rank Category 3, or blue-green algae cells > 100k/ml: POSTED BEACH CLOSURE (If public has beach access, alert water users that a blue-green algae bloom is present), POSTED ADVISORY (At other impacted access points)
Idaho	Surface scum visible and associated with toxigenic taxa* Sum of all potentially toxigenic taxa ≥100,000 cells/mL The density of Microcystis or Planktothrix >40,000 cells/mL <small>*Toxigenic taxa include Anabaena, Microcystis, Planktothrix, Nostoc, Coelosphaerium, Anabaenopsis, Aphanizomenon, Gloeotrichia, Woronichinia, Oscillatoria, and Lyngbya. Additional taxa are known to be potentially toxic and may be added to the list.</small>	Recommend posting by Public Health District (PHD) in conjunction with water body management agency Recommend posting by PHD in conjunction with water body operator Recommend posting by PHD in conjunction with water body operator

Illinois	Microcystin-LR concentration results approach or exceed 10 µg/L	Reporter of HAB event and the local lake management entity will be informed immediately.
Indiana	Level 1: very low/no risk < 4 µg/L microcystin-LR Level 2: low to moderate risk 4 to 20 µg/L microcystin-LR Level 3: serious risk > 20 µg/L microcystin-LR Warning Level: Cylindrospermopsin: 5 ppb	Level 1: use common sense practices Level 2: reduce recreational contact with water Level 3: consider avoiding contact with water until levels of toxin decrease
Iowa	Microcystin ≥ 20 µg/L	Caution - bloom present no toxin data available Warning - when toxin levels exceed 20 µg/L
Kansas	PHA: >4 µg/L to <20 µg/L for microcystin or > 20,000 cell/mL to <100,000 cell/mL cyanobacteria cell counts PHW: > 20 µg/L or > 100,000 cell/mL cyanobacterial cell counts and visible scum present	Public Health Advisory (PHA): avoid contact Public Health Warning (PHW): all contact with water is restricted
Kentucky (Louisville District)	Advisory: >20,000 cells/mL of cyanobacteria cell counts Caution: > 100,000 cells/mL of cyanobacteria cell counts	Advisory: contact discourage, water may be unsafe Caution: Closure, contact prohibited
Massachusetts	14 µg/L for microcystin-LR and ≥ 70,000 cells/mL for cyanobacteria cell counts	Advisory - Avoid contact with water
Nebraska	Microcystin ≥ 20 µg/L	Health Alert
New Hampshire	>50% of cell counts from toxigenic cyanobacteria	Public Health Advisory


State Guidelines—Recreational Water

(just a few shown)

New York	Suspicious Bloom: Visual appearance of a bloom likely to be cyanobacteria Confirmed Bloom: Blue green chlorophyll levels $\geq 25 \mu\text{g/L}$ and microscopic confirmation that majority of sample is cyanobacteria or report of closure of a regulated swimming area Confirmed with High Toxins Bloom: Confirmed Bloom and microcystin $\geq 20 \mu\text{g/L}$ (shoreline samples) or microcystin $\geq 10 \mu\text{g/L}$ (open water samples) or known risk of exposure to anatoxin or another cyanotoxin, based on consult between NYSDEC HABs Program and NYSDOH staff.	Notifications will be updated weekly all summer and into the fall For all blooms, avoid exposure. Keep children and pets away from scums or discolored water Seek immediate medical assistance for symptoms consistent with exposure Report any symptoms to local/state Health Department Report blooms to DEC
North Carolina	Visible discoloration of the water or a surface scum may be considered for microcystin testing	Advisory/Closure
Ohio	Microcystin-LR: PHA: $6 \mu\text{g/L}$; NCA: $20 \mu\text{g/L}$ Anatoxin-a: PHA: $80 \mu\text{g/L}$; NCA: $300 \mu\text{g/L}$ Saxitoxin: PHA: $0.8 \mu\text{g/L}$; NCA: $3 \mu\text{g/L}$ Cylindrospermopsin: PHA: $5 \mu\text{g/L}$; NCA: $20 \mu\text{g/L}$	Public Health Advisory (PHA) - swimming and wading are not recommended, water should not be swallowed and surface scum should be avoided. No Contact Advisory (NCA) -recommend the public avoid all contact with the water
Oklahoma	$100,000 \text{ cell/mL}$ of cyanobacteria cell counts and $> 20 \mu\text{g/L}$ for microcystin	Blue-Green Algae Awareness Level Advisory
Oregon	Option 1: Visible scum and cell count or toxicity Option 2: Toxigenic species $>100,000 \text{ cells/mL}$ Option 3: Microcystis or Planktothrix $> 40,000 \text{ cells/mL}$ Option 4: Toxin Testing Microcystin: $10 \mu\text{g/L}$ Anatoxin-a: $20 \mu\text{g/L}$ Cylindrospermopsin: $6 \mu\text{g/L}$ Saxitoxin: $100 \mu\text{g/L}$	Public Health Advisory
Rhode Island	Visible cyanobacteria scum or mat and/or cyanobacteria cell count $> 70,000 \text{ cells/mL}$ and/or $\geq 14 \mu\text{g/L}$ of microcystin-LR	Health Advisories
Texas	$>100,000 \text{ cell/mL}$ of cyanobacteria cell counts and $>20 \mu\text{g/L}$ microcystin	Blue-Green Algae Awareness Level Advisory


Vermont	Close and post any public beach if any of the following conditions are met: <ul style="list-style-type: none"> Visible known blue-green algae bloom/scum or an unknown, potentially blue-green algae (i.e., not pollen), bloom/scum Microcystin-LR (equivalents) concentration greater than or equal to $6 \mu\text{g/L}$ micrograms per Liter or parts per billion (ppb) Anatoxin-a concentration greater than or equal to $10 \mu\text{g/L}$ (ppb) Cylindrospermopsin concentration greater than or equal to $10 \mu\text{g/L}$ (ppb) 	Beach Closure
Virginia	$5,000$ to $<20,000$ Microcystis cells/mL $20,000$ to $100,000$ Microcystis cells/mL $> 100,000$ Microcystis cells /mL, or $> 6 \mu\text{g/L}$ microcystin concentration, or Blue-green algal "scum" or "mats" on water surface	Local agency notification; initiate bi-weekly water sampling Public notification indicating a harmful algal bloom is present in recreational water; initiate weekly sampling Immediate public notification to avoid all recreational water contact where bloom is present; continue weekly sampling
Washington	Microcystin-LR: $6 \mu\text{g/L}$ Anatoxin-a: $1 \mu\text{g/L}$ Cylindrospermopsin: $4.5 \mu\text{g/L}$ Saxitoxin: $75 \mu\text{g/L}$	Tier 1. Caution: when a bloom is forming or a bloom scum is visible (toxic algae may be present) Tier 2. Warning: Toxic algae present Tier 3. Danger: Lake closed
Wisconsin	$> 100,000 \text{ cells/mL}$ or scum layer	Advisory/Closure

Guidelines—EPA May 2019



United States Environmental Protection Agency Office of Water Mail Code 4304T EPA 822-R-19-001 May 2019

Recommended Human Health Recreational Ambient Water Quality Criteria or Swimming Advisories for Microcystins and Cylindrospermopsin



United States Environmental Protection Agency Office of Water EPA 822-F-19-001 May 2019

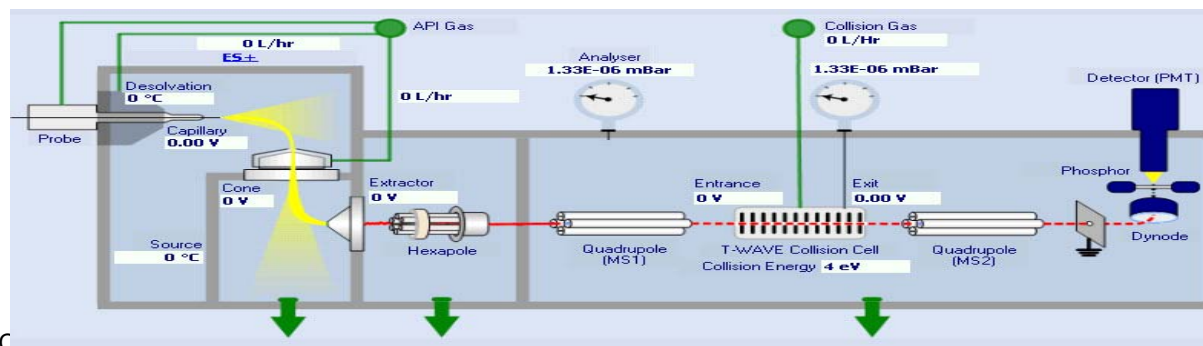
Recommended Human Health Recreational Ambient Water Quality Criteria or Swimming Advisories for Microcystins and Cylindrospermopsin

Table. Recommended magnitude for cyanotoxins.

Microcystins	Cylindrospermopsin
8 µg/L	15 µg/L

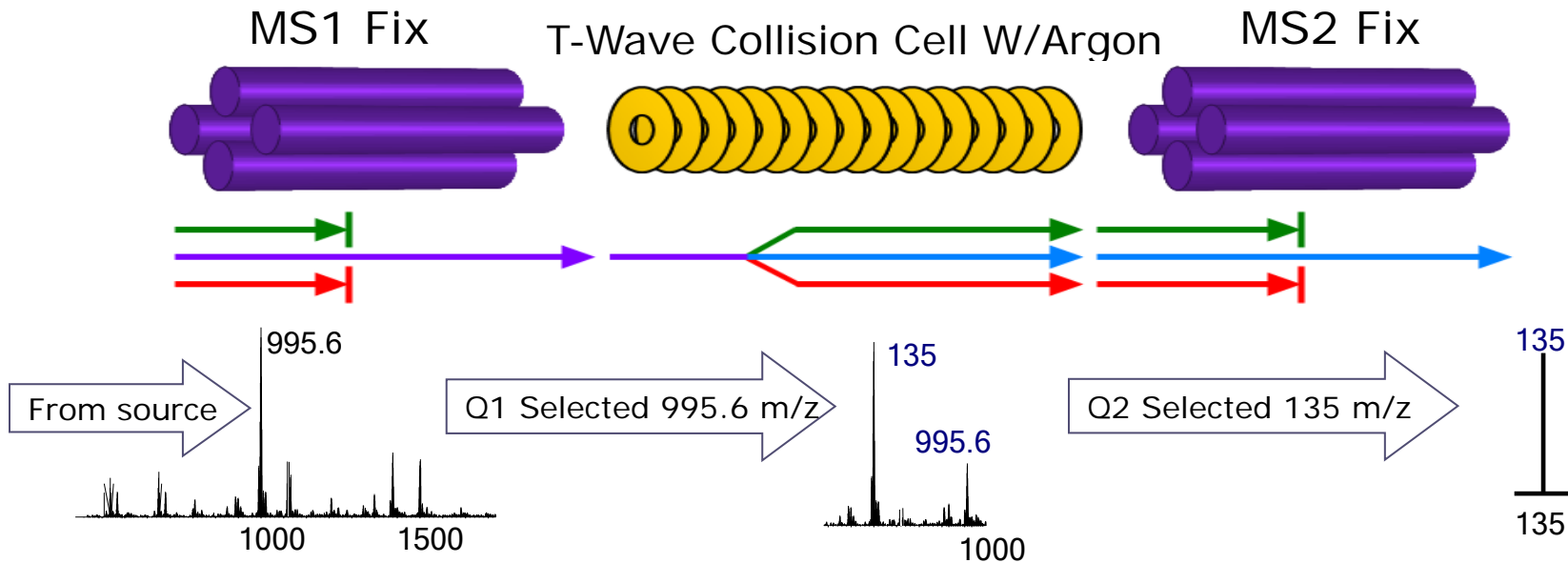
What is a Tandem Quadrupole MS?

- Tandem Quadrupoles consist of 2 mass analysers (quads), and a collision cell, enabling **more selective** analyses to be performed compared to a single quadrupole or other non MS detection mode.
- Their selectivity has made them the industry standard for MS based quantitation.
 - The ions generated in the source are filtered by the **first mass analyser**.
 - A **collision cell** can then be used to **fragment** molecules exiting the first mass analyser.
 - These fragments are then filtered by the **second mass analyser**.



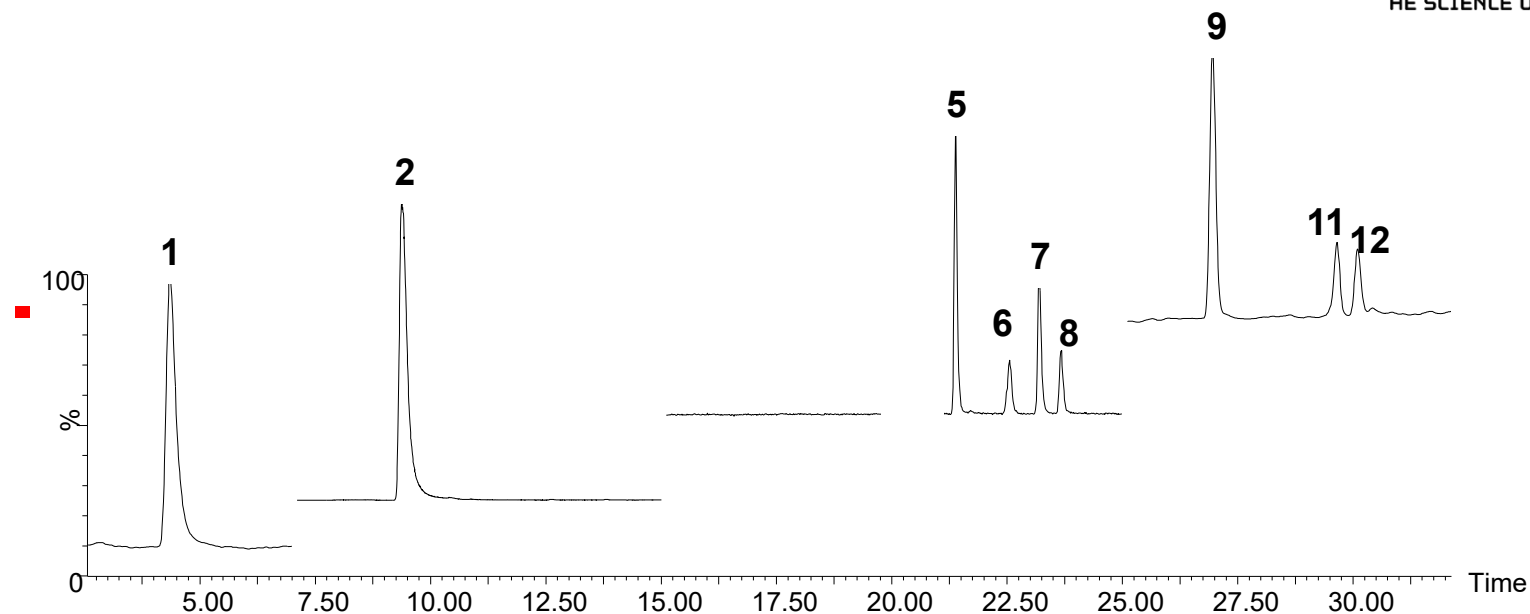
Tandem Experiments

Multiple Reaction Monitoring (MRM)



- The system is set up for selectivity, allowing only a selected product ion to be fragmented and one fragment ion to be detected.
- Multiple MRM's can also be use, as well as several fragments from a specified product ion for confirmation purposes.

Separation using Traditional HPLC/MS/MS



1=Cylindrospermopsin, 2=Anatoxin-a, 5= Microcystin RR, 6=Nodularin, 7=Microcystin YR, 8=Microcystin LR, 9=Microcystin LA, 11=Microcystin LW, and 12=Microcystin LF

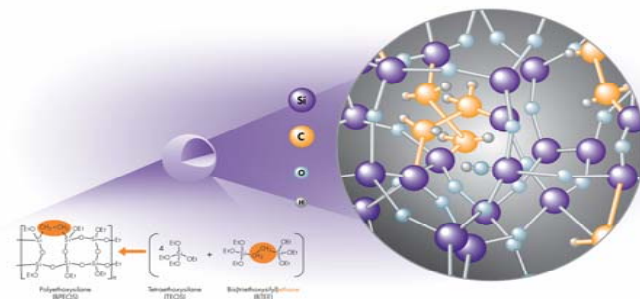
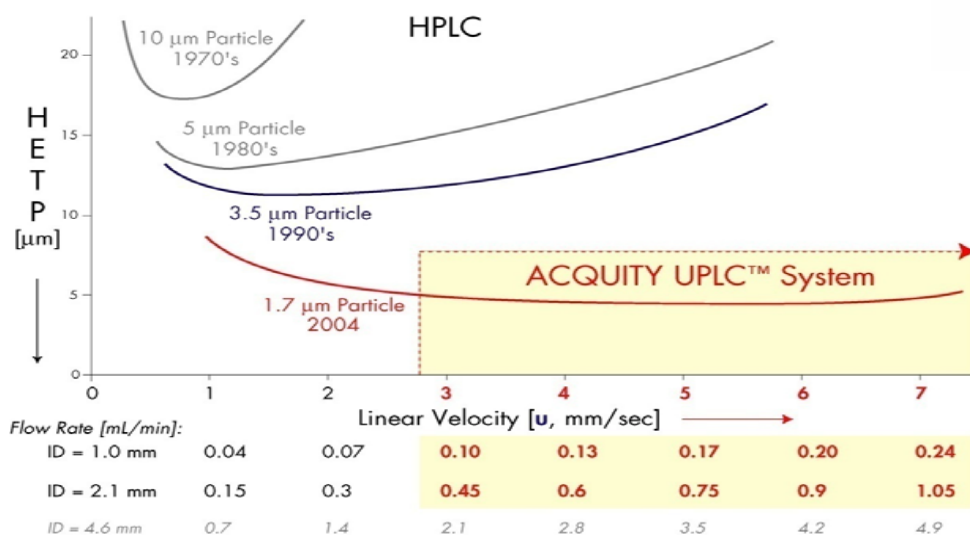
2.1X150mm Atlantis dC₁₈ (3.5µm)@30C-0.29mL/min

Going the Next Step...UltraPerformance LC/MS/MS

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- Small Particle (sub 2µm)
- Higher separation power
- Higher tensile strength



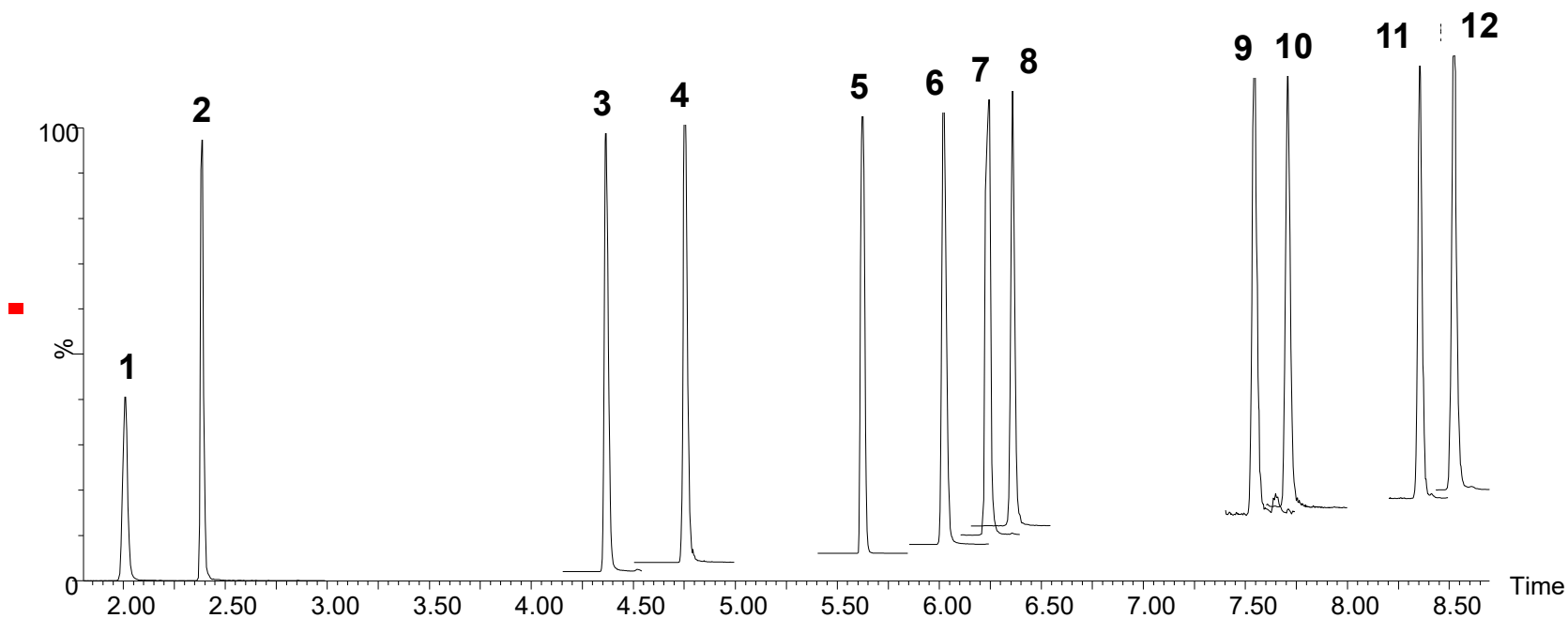
Xevo TQ-S micro

Acquity™
 Ultra Performance LC

UPLC™/MS/MS Separation

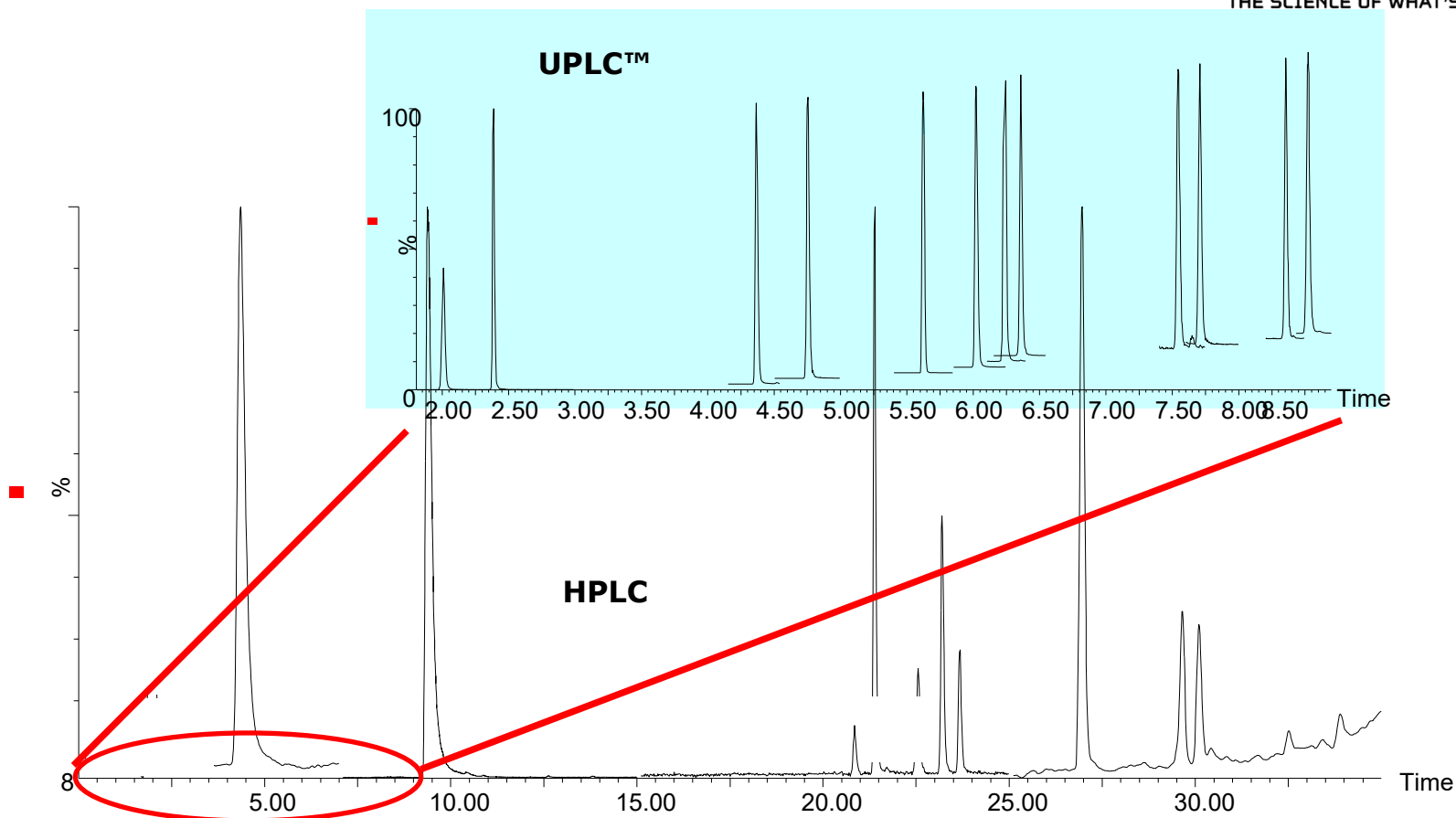
8.5 minutes (HSS T3 2.1x100mm (1.8um))

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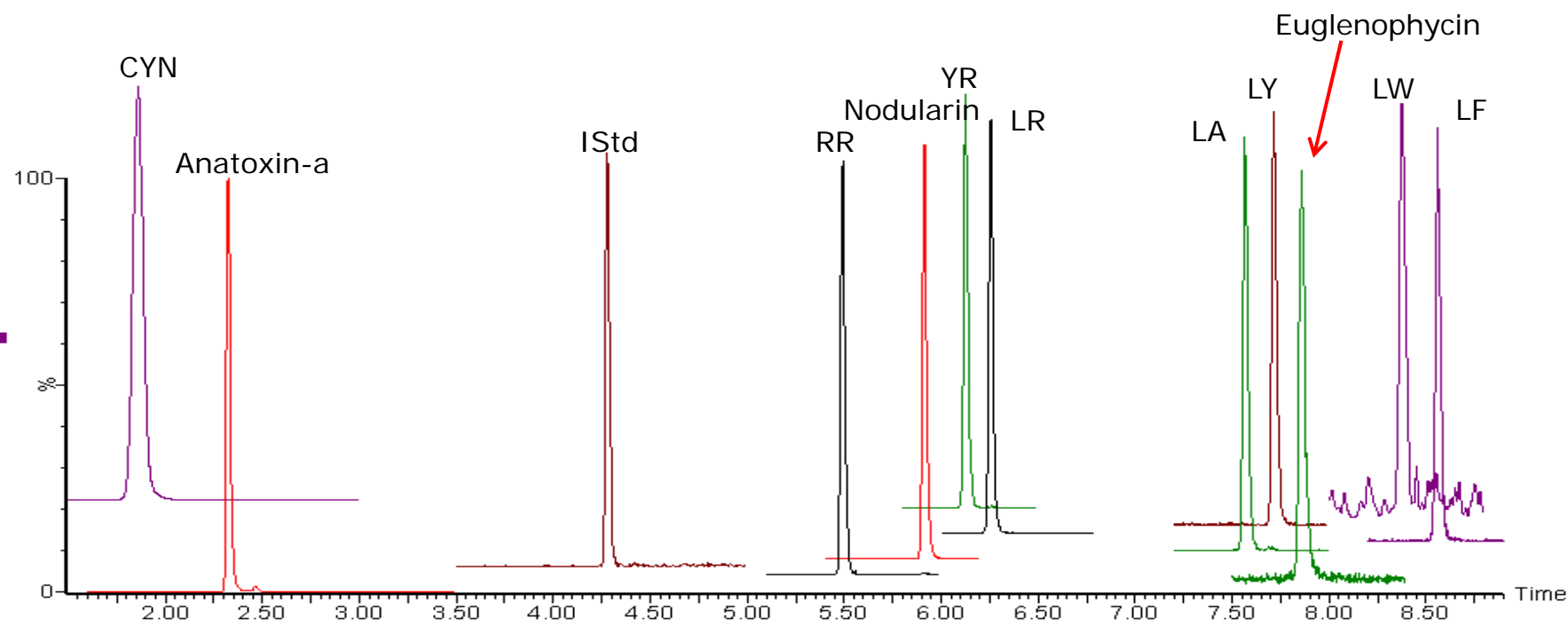
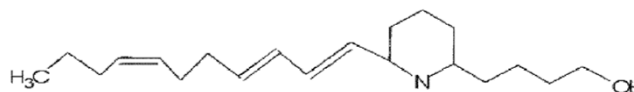


1=Cylindrospermopsin, **2**=Anatoxin-a, **3**=Cyclo (Arg-Ala-Asp-D-Phe-Val) (IStd), **4**=[Leu⁵]-Enkephalin (IStd), **5**= Microcystin RR, **6**=Nodularin, **7**=Microcystin YR, **8**=Microcystin LR, **9**=Microcystin LA, **10**=Microcystin LY, **11**=Microcystin LW, and **12**=Microcystin LF

HPLC vs UPLC™

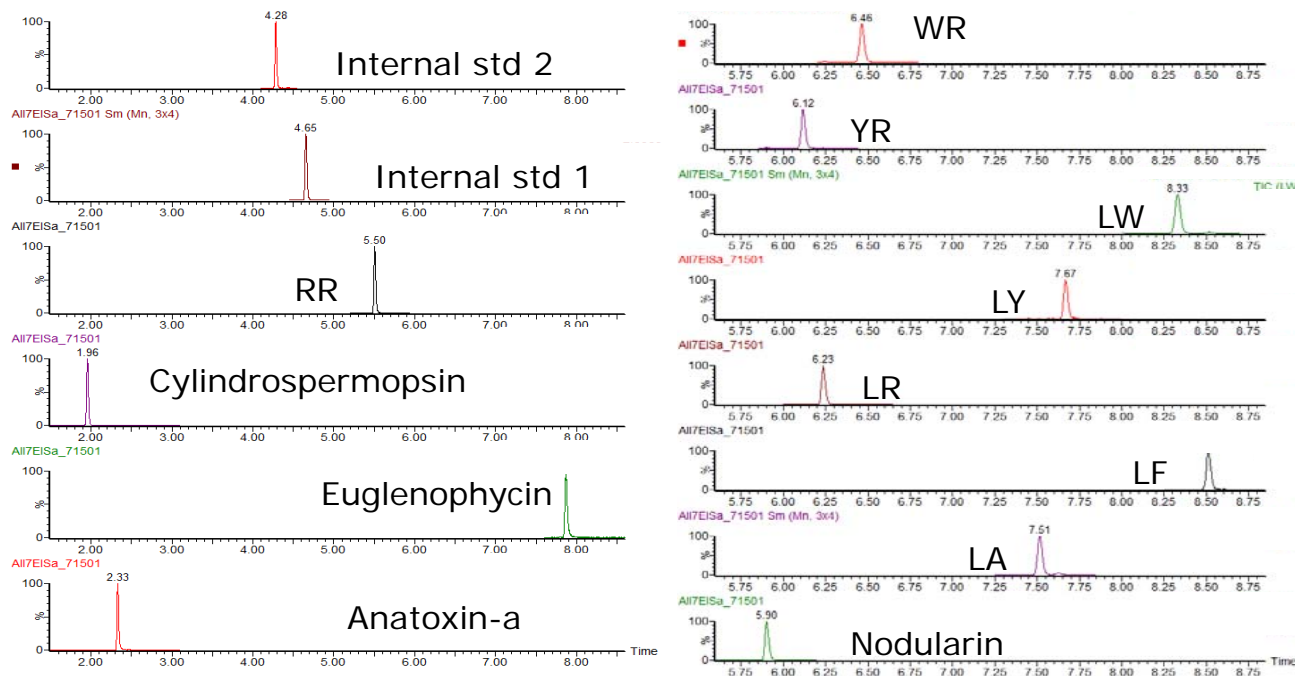


Additional Toxin (Euglenophycin) from *Euglena sanguinea*



Analysis done using UPLC/MS/MS method (Expanded!)

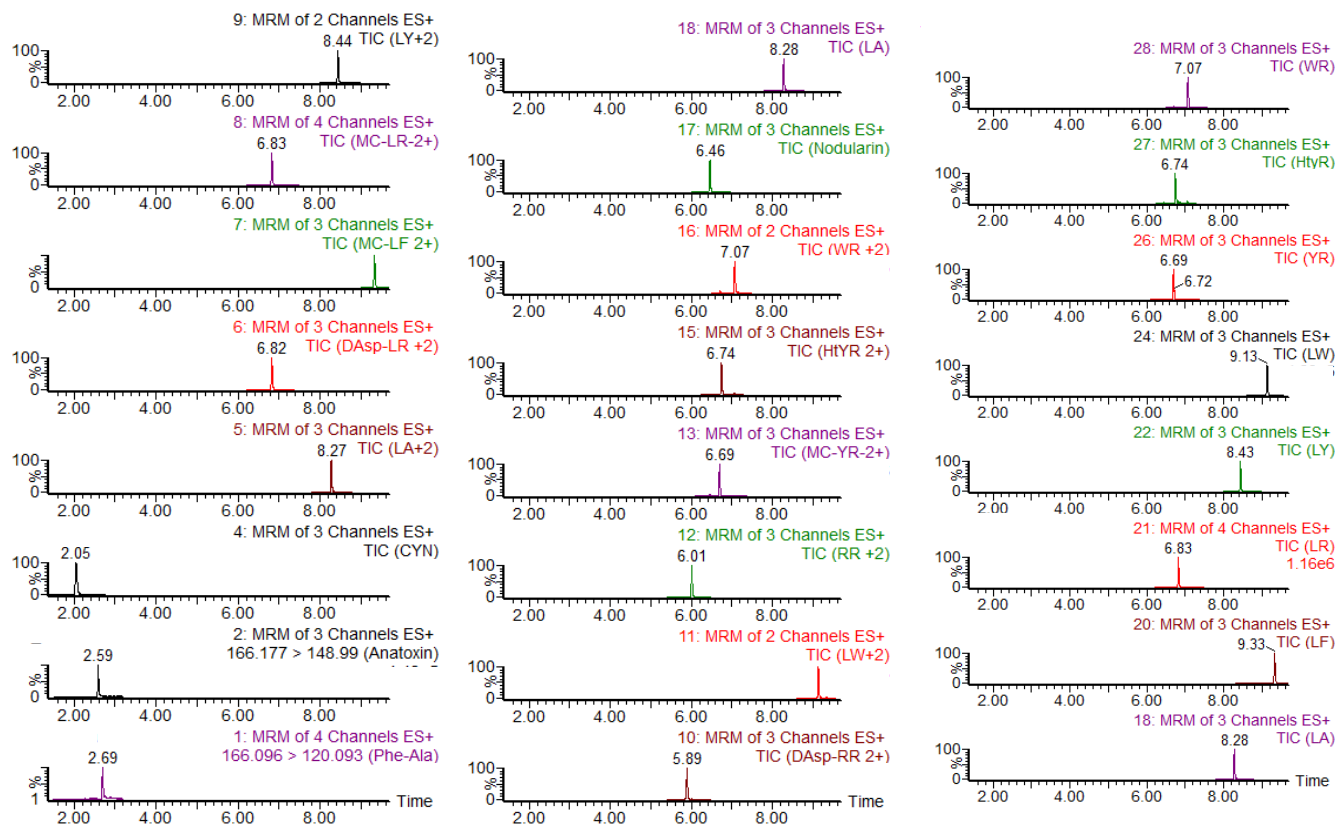
- Allows for screening of microcystins, anatoxin, euglenophycin, nodularin, nodularin, anabaenopeptins and cylindrospermopsin (2 internal Standards) in a <10 minute run



Expanded List

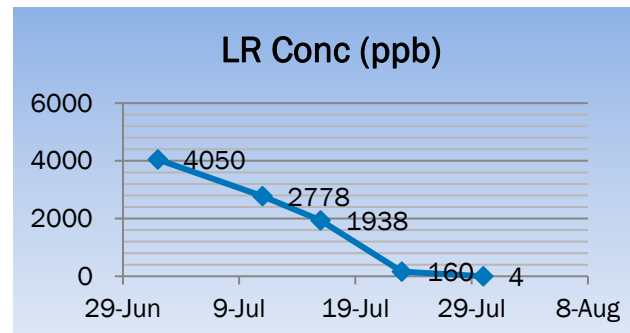
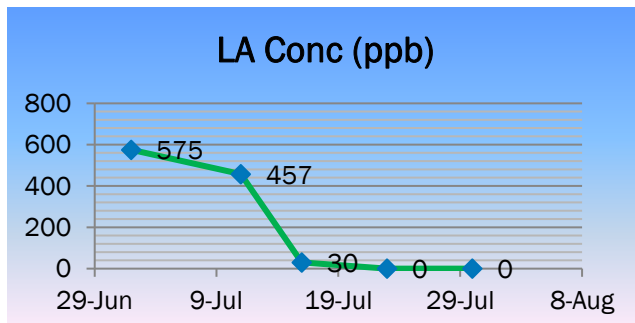
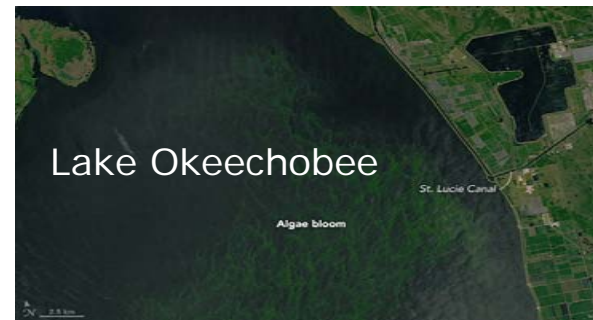
LR	Phe-Ala
RR	Anabaenopeptin A
YR	Anabaenopeptin B
LA	Cylindrospermopsin
LF	Anatoxin
LY	Nodularin
LW	Euglenophycin
WR	Ethylated MC-LR (d5)(IS)
HtYR	PI-Cylco (IS)
D-Asp3-RR	Leu-Enk (IS)
D-Asp3-LR	Micropeptin 1106
D-Asp3-Dhb7-MC-Htyr	Aeruginosamide B

Current Separation (ESI/MS/MS)



Toxin Incidents

- Florida July 2016...Martin County, Florida*



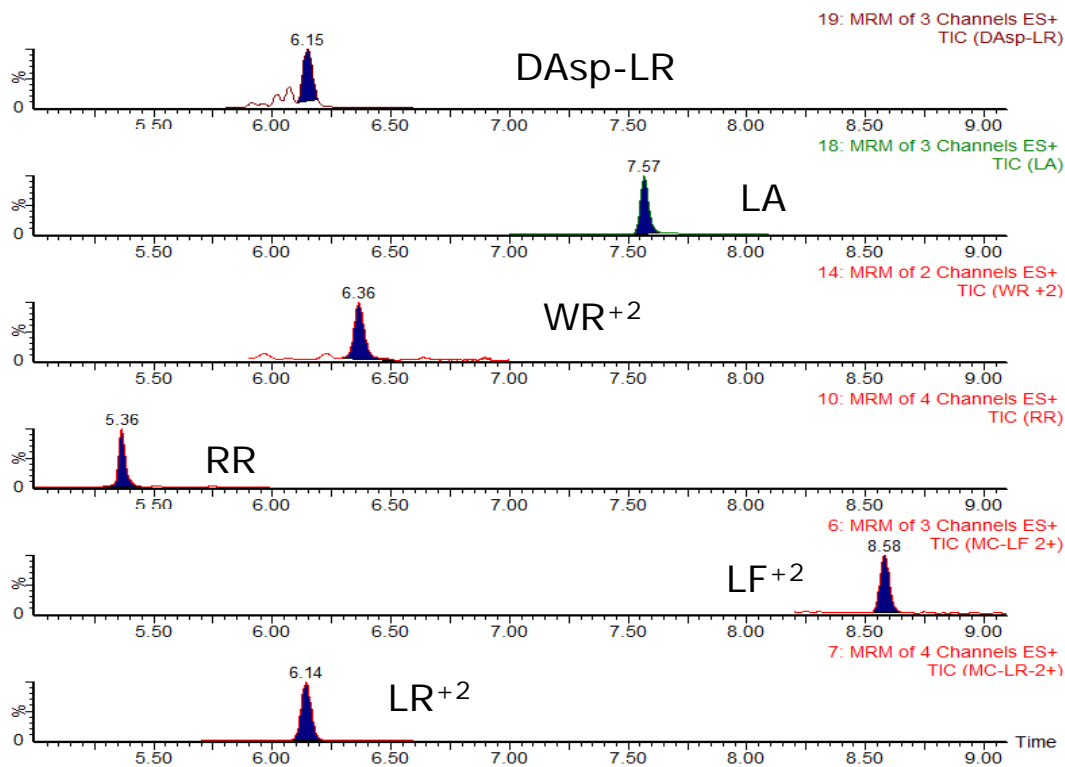
***"Toxin composition of the 2016 *Microcystis aeruginosa* bloom in the St. Lucie Estuary, Florida"**

Oehrle S, Rodriguez-Matos M, Cartamil M, Zavala C, Rein KS.
Toxicon. 2017 Nov; vol.138: p.169-172.

Example Chromatogram--Florida

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	ppb
LR	2600
RR	6
WR	7
DAsp-LR	120
LF	5
LA	632



Microcystis aeruginosa

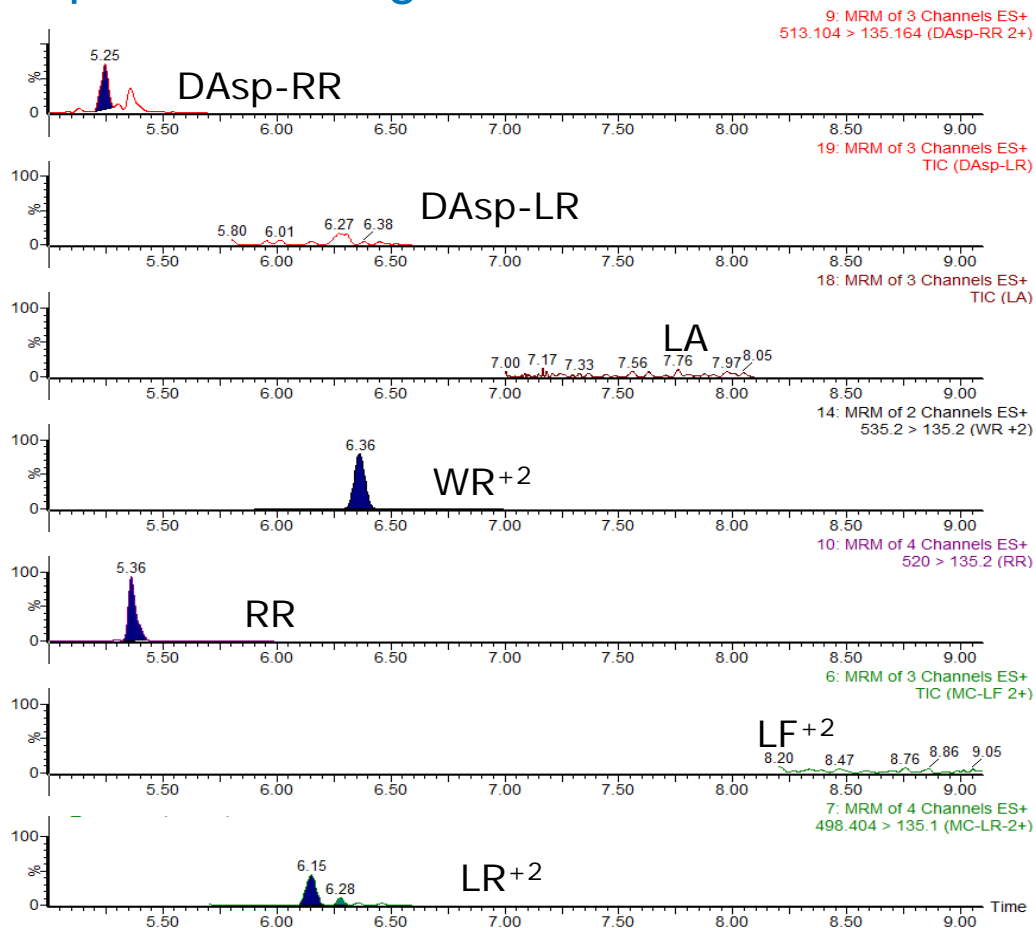
Additional Toxin Incidents

- California August-September 2016...Lake Elsinore



Example Chromatogram—Lake Elsinore

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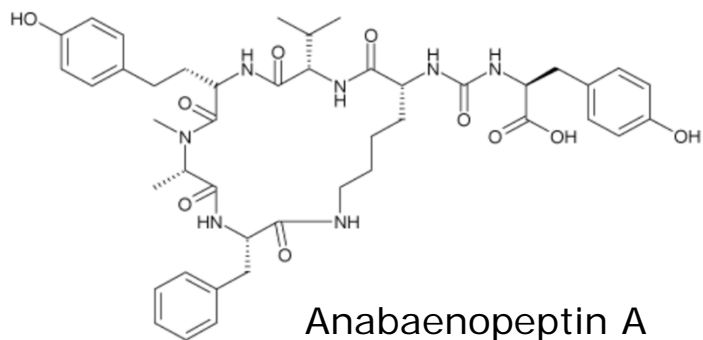
Microcystis flos-aquae

	ppb
LR	6
RR	7671
WR	2628
DAsp-LR	ND
LF	ND
LA	ND
DAsp-RR	142



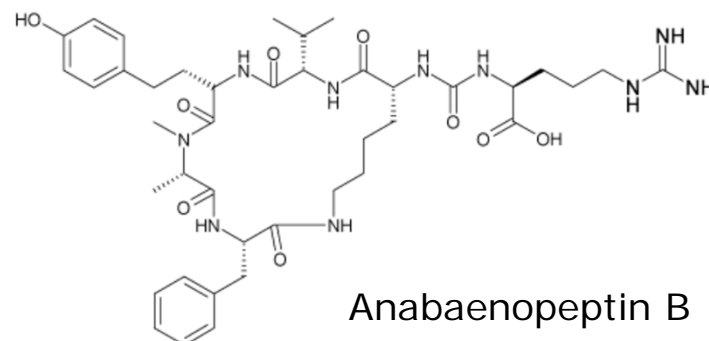
Going even FARTHER! (adding Anabaenopeptin toxins)

- Anabaenopeptins are a highly diverse group of bioactive peptides produced by several genera of cyanobacteria such as *Anabaena*, *Planktothrix*, *Microcystis* and *Nodularia*. These peptides are commonly detected in cyanobacterial blooms along with the well known microcystins.



Anabaenopeptin A

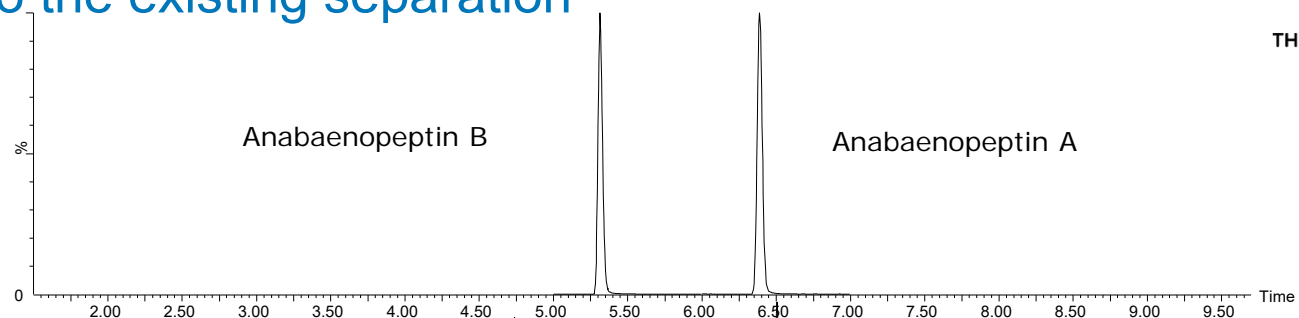
FW=843.9



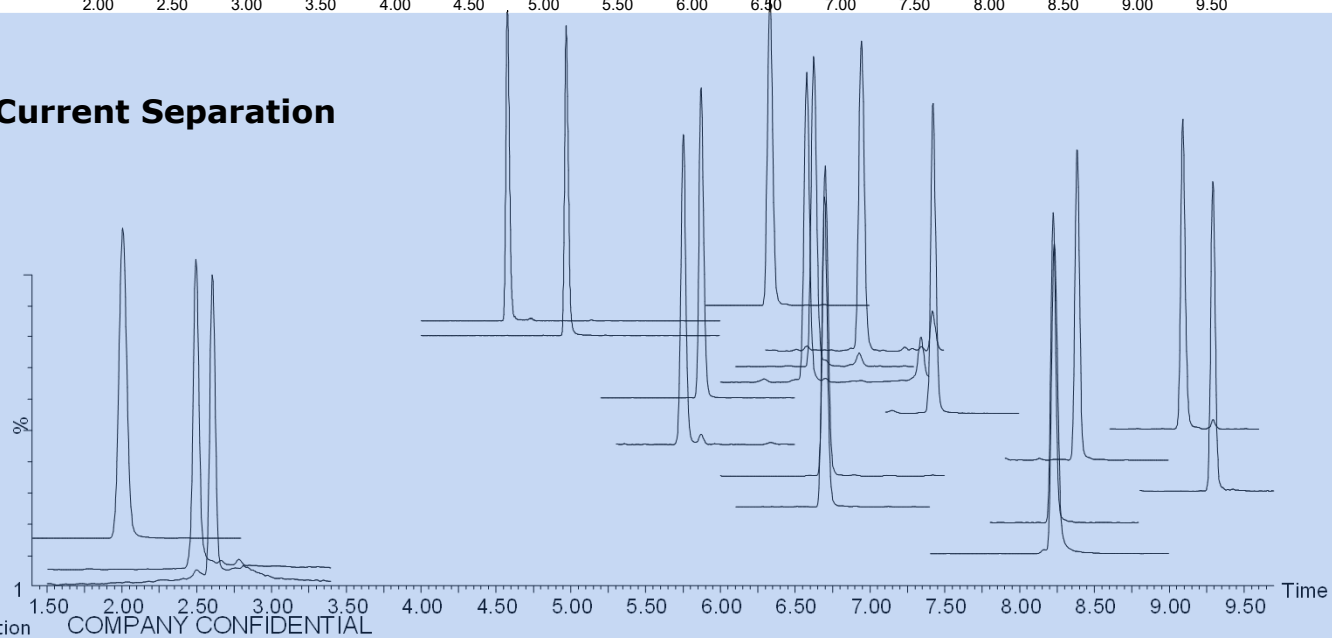
Anabaenopeptin B

FW=837

Added to the existing separation

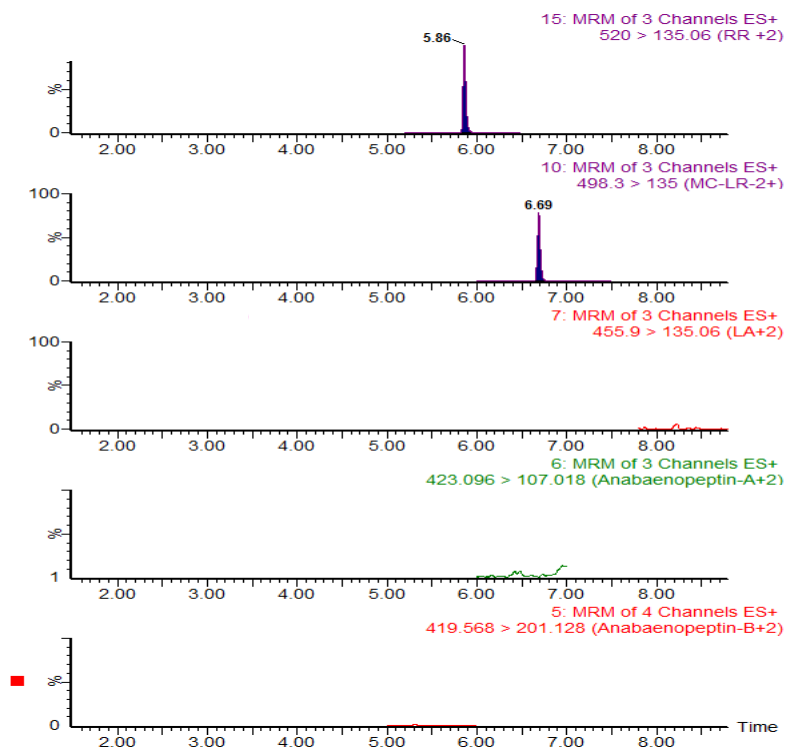


Current Separation

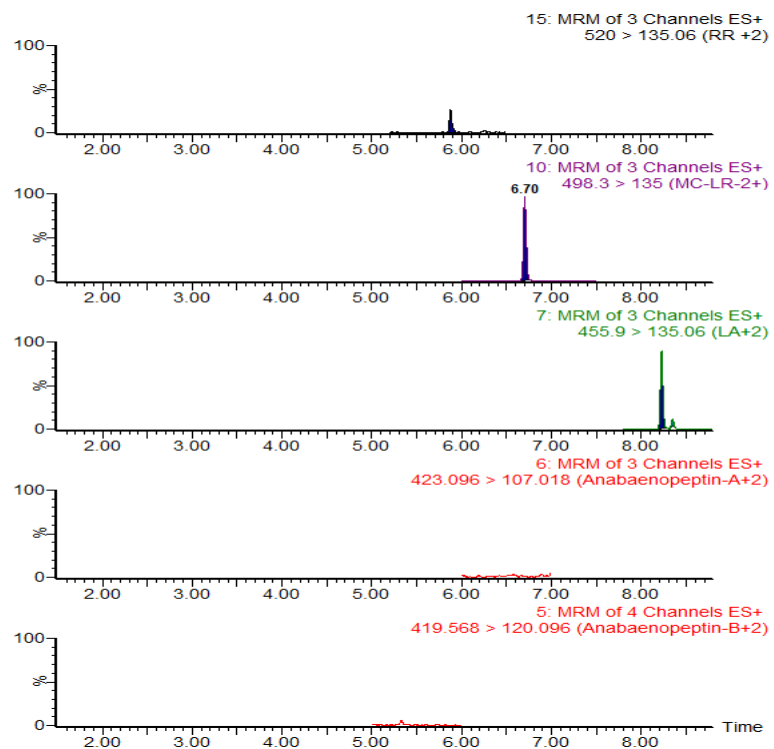


Additional Samples—July 2018

Lake Sample (KY)

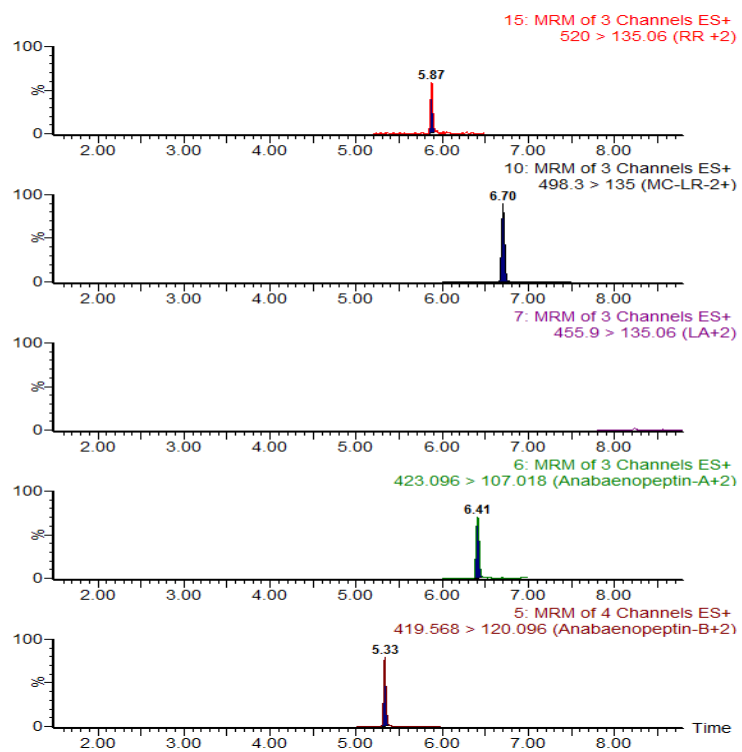


Lake Sample (Western US)



Additional Samples—July 2018

Lake Sample 2 (Western US)



August 2019

- Dog Deaths in Wilmington, NC pond

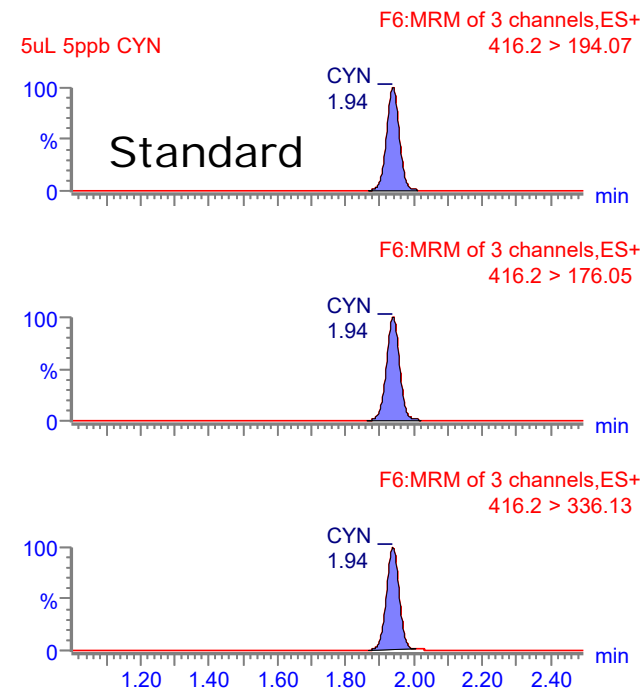
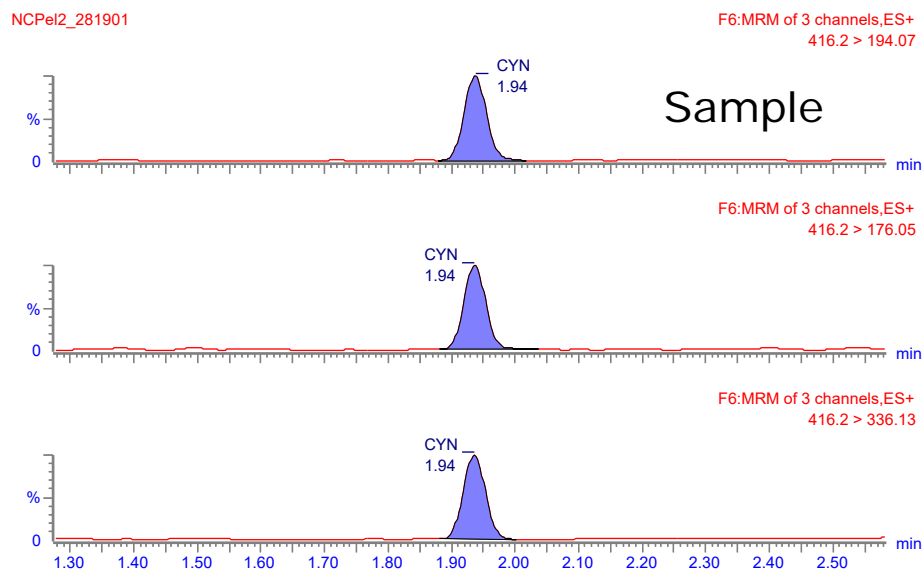


Benthic Algae produced

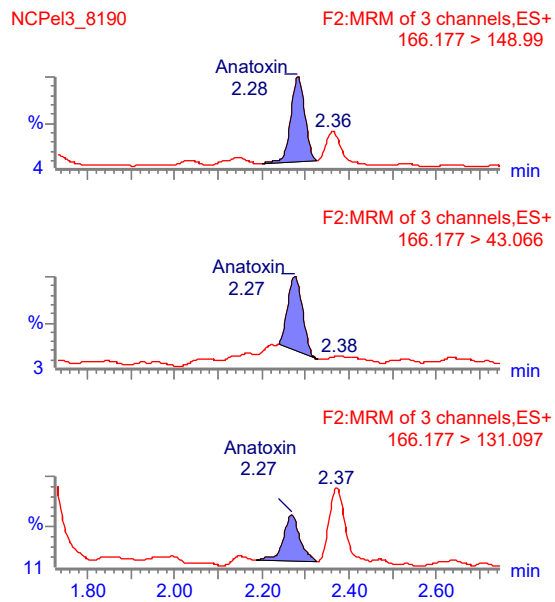
Primary strain *Oscillatoria*

LC/MS/MS Analysis

- Samples Frozen and shipped
- 2 water samples and a concentrate (pellet)

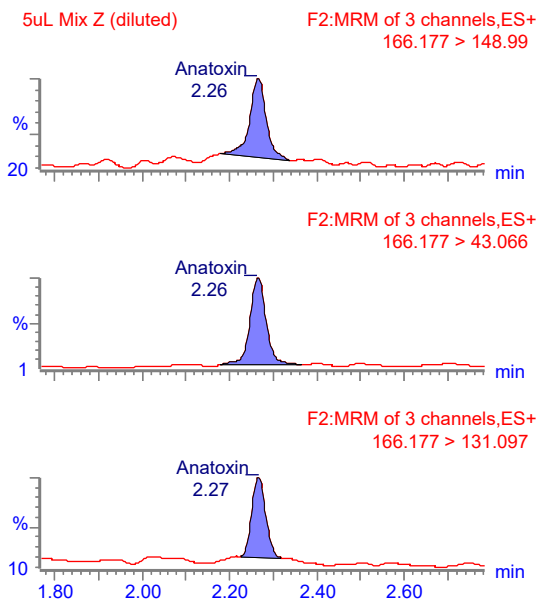


NC Pellet



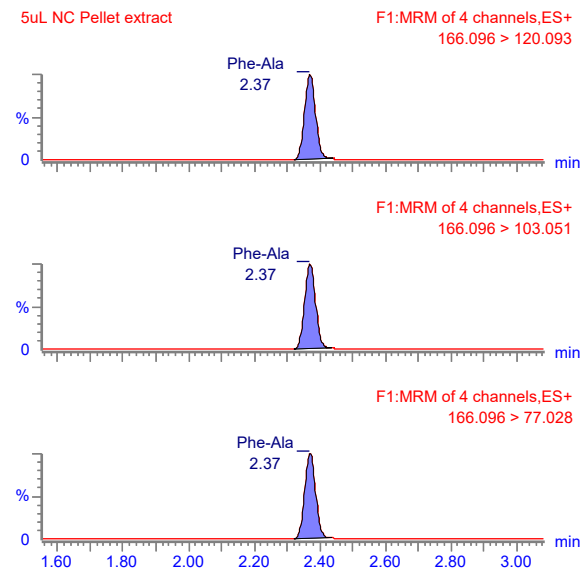
Anatoxin

Standard



Anatoxin

NC Pellet

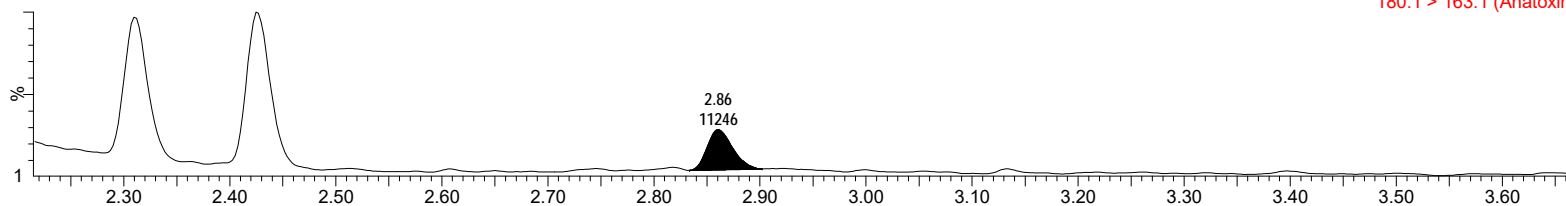


Phe-Ala

Initial screen-Homoanatoxin

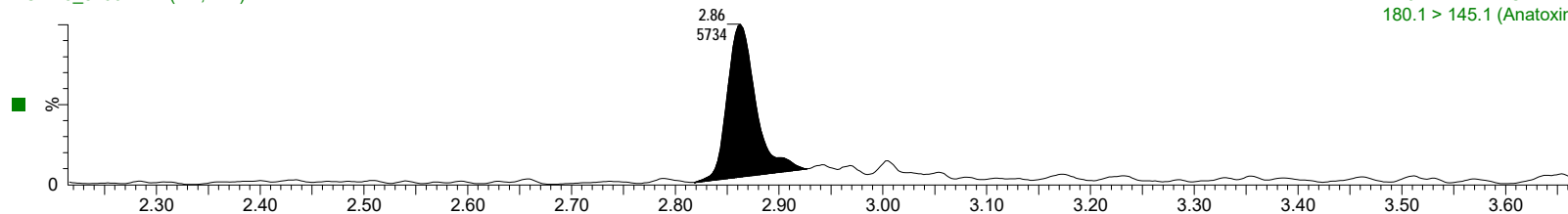
5uL NC Pellet extract (conc) (8/19)--2.1X100mm HSS-T3

3: MRM of 7 Channels ES+
180.1 > 163.1 (Anatoxin-homo)



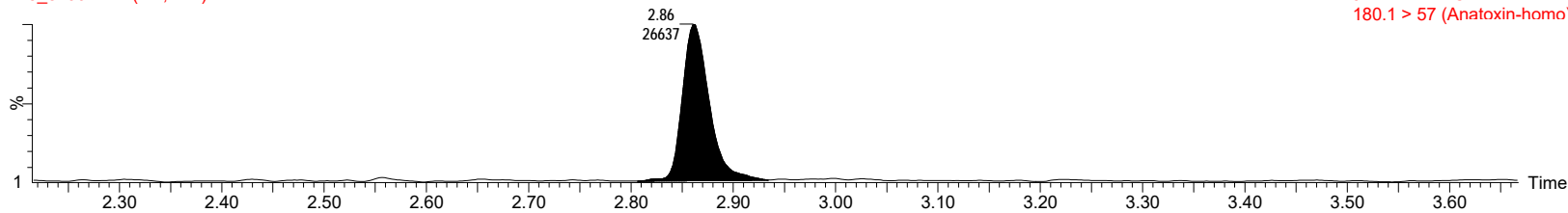
NCPel3_81901 Sm (Mn, 2x2)

3: MRM of 7 Channels ES+
180.1 > 145.1 (Anatoxin-homo)

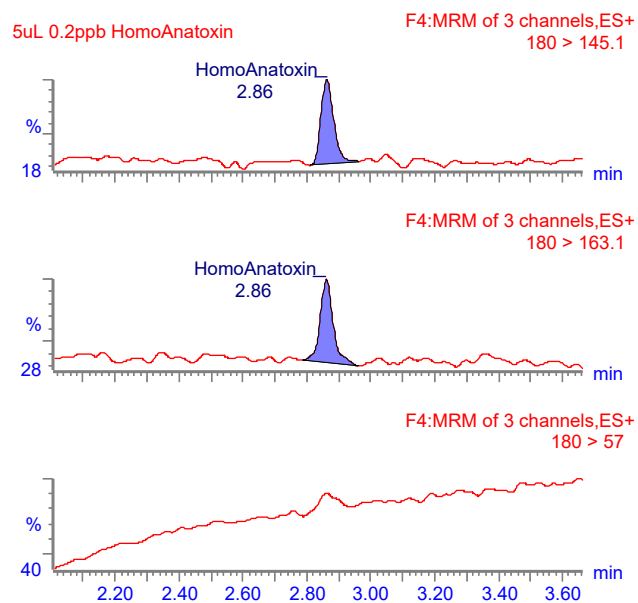


NCPel3_81901 Sm (Mn, 2x2)

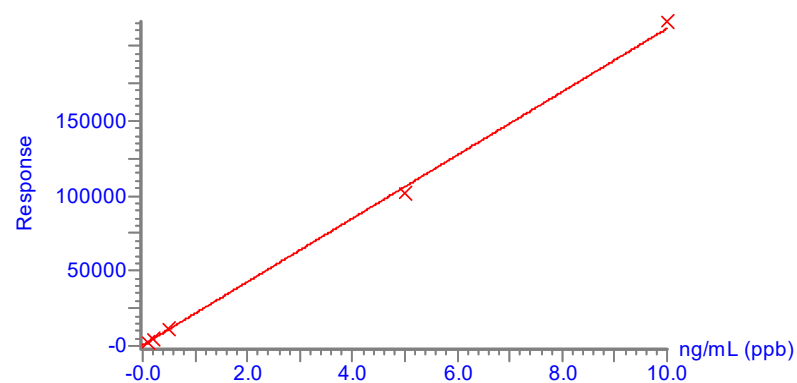
3: MRM of 7 Channels ES+
180.1 > 57 (Anatoxin-homo)



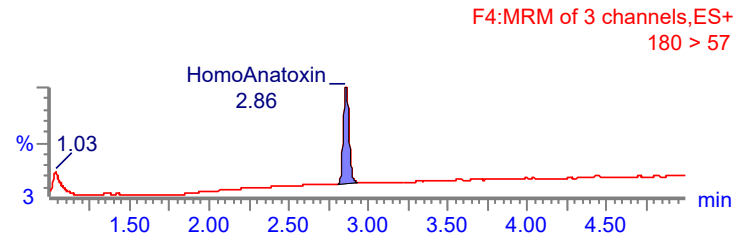
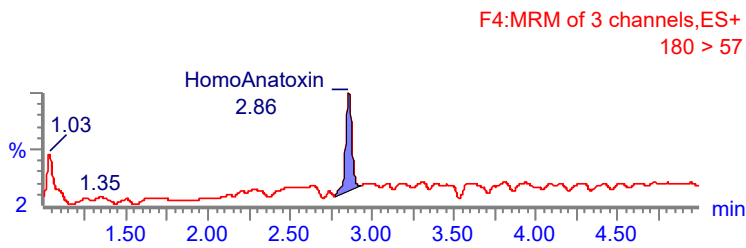
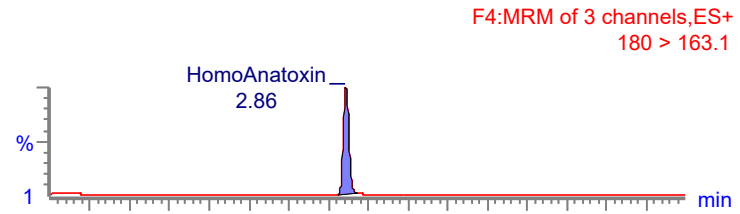
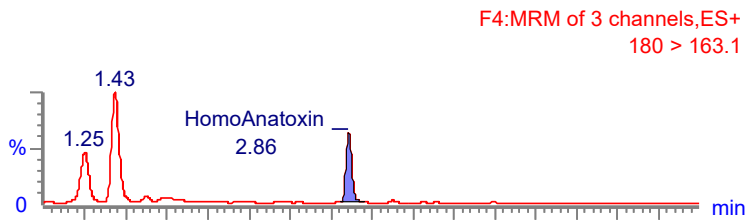
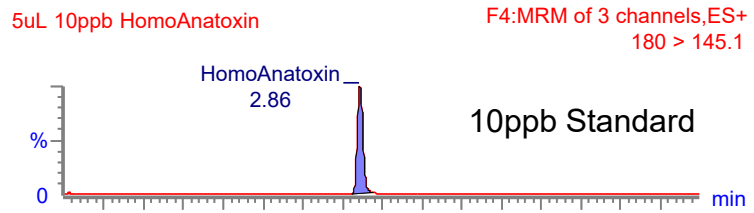
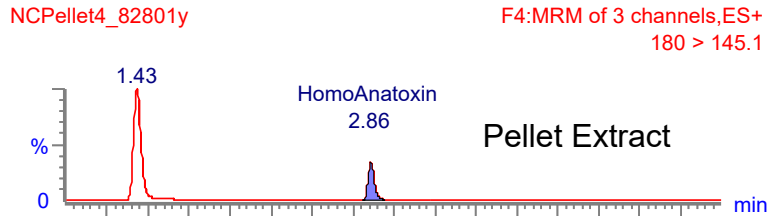
Homoanatoxin Standard (0.2ppb)



Compound name: HomoAnatoxin
Correlation coefficient: $r = 0.999407$, $r^2 = 0.998814$
Calibration curve: $21114 * x + 476.17$
Response type: External Std, Area
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



Sample and Standard (Homoanatoxin)



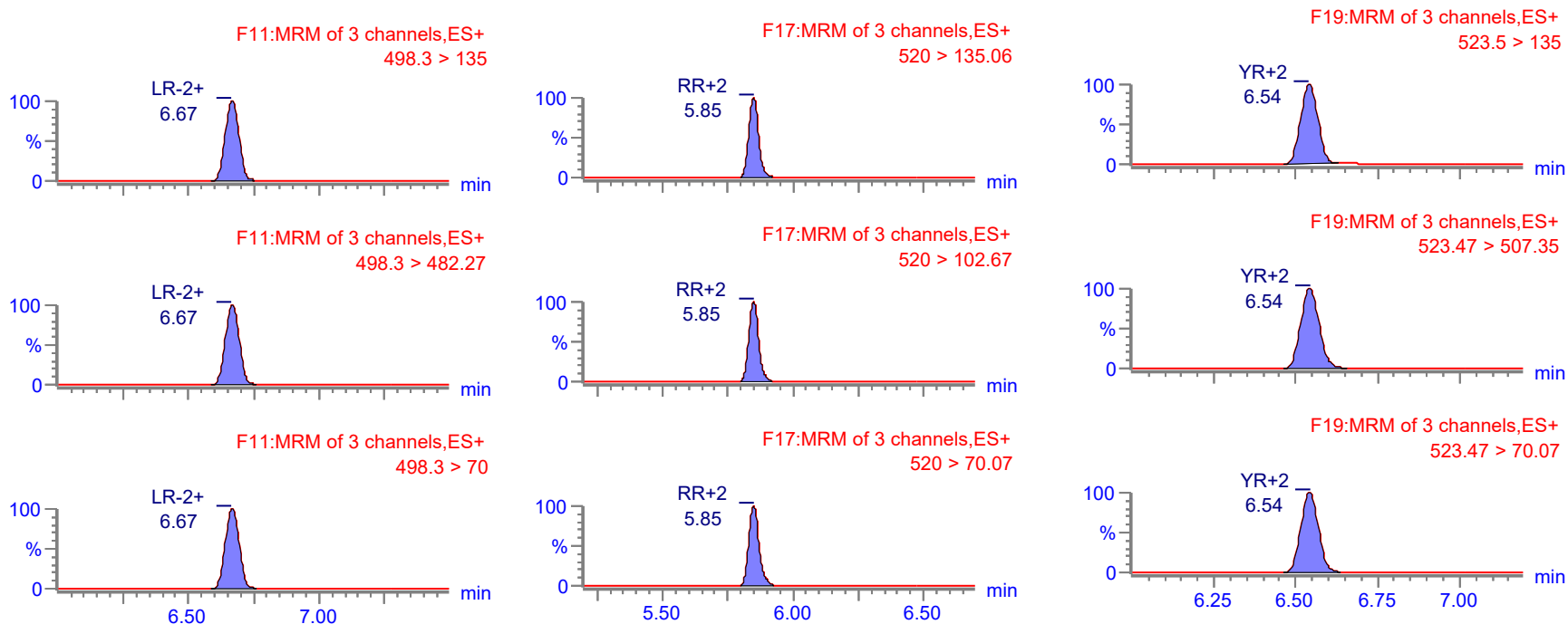
Ohio River Bloom (September-October 2019)

Waters

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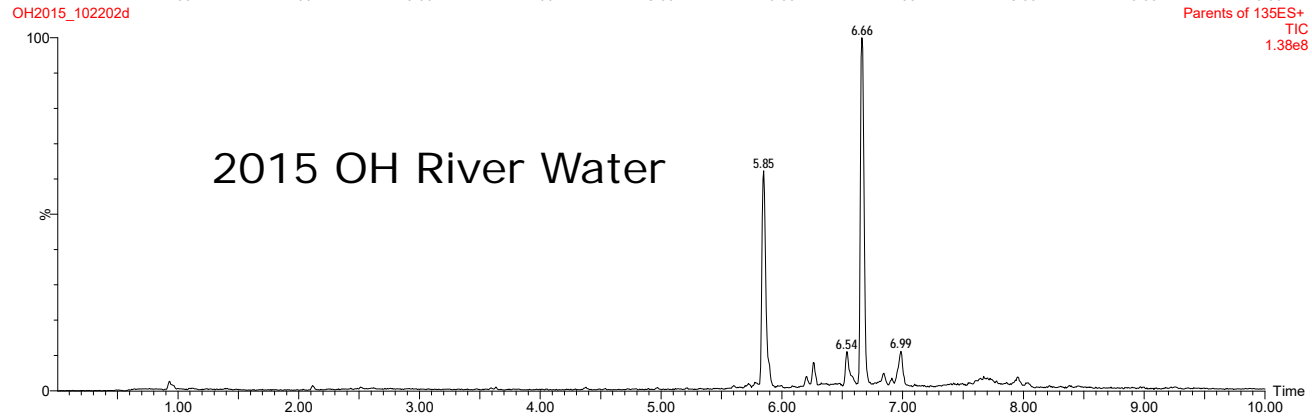
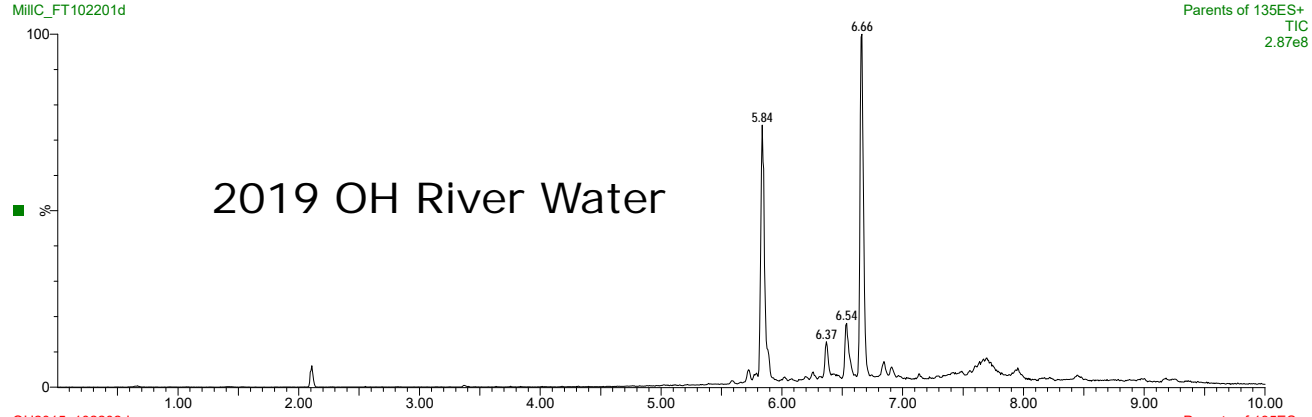
Results (freeze/thaw)



Also DAsp-LR, DAsp-RR, and Anabaenopeptin B

Interesting Screen (Precursor Scan (135 (ADDA)))

5uL OH River at Mill Creek (10/19/19)(freeze/thaw)
MillC_FT102201d



Summary

- Mass Spectrometry offers a sensitive and selective method to detect various toxins.
- UPLC offers high resolution and fast analysis times. (real time decisions).
- MS/MS technology can also be used for other critical/emerging water assays (pesticides, PFOS, and Persistent organic pollutants (POP's) for example).

Acknowledgements

- Waters Corporation
- Thomas More College Biology Field Station
- NKU Biology Department...Dr. Miriam Kannan
- Dr. Kelly Rein and Research group at FIU
- Catharina Alves-de-Souza and Wendy Strangman...UNC-Wilmington

Take Home Message

- As Captain Jack Sparrow said



Waters
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“When swimming.....Drink Rum...not the water!”

Thank You for Attending

Waters
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