Targeted Toxin Analysis….Beyond the Compounds in EPA Method 544 and 545..Be careful what you look for..it might be there.

Stuart Oehrle

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Abstract

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- Cyanotoxin analysis of various water sources (recreational and intake for drinking) is critical from a safety standpoint. Targeted LC/MS/MS analysis is a particularly useful technique to monitor for algal toxins. These analyses are generally guided by commercial standards, which are available for a suite of common toxins. However, hundreds of toxin variants exist and most, including toxins that have been seen regularly in blooms, do not have established, standards or reference material. Since EPA Methods 544 and 545 have come out in 2015 several toxin standards have become available. In addition, many “other” compounds of interest have arisen as both standards and materials become available. This talk will discuss specific examples, including results from intake water samples as well as direct injection analysis of sub ppb levels of the standard toxins in drinking water intakes from a river. Expanded monitoring for toxins in a single injection will be discussed.

Algal Toxins

- These toxins are typically grouped by their main mode of action, such as:
  - Hepatotoxins (toxins that damage the liver)
  - Dermatoxins (toxins that damage the skin)
  - Neurotoxins (toxins that damage the nerve cells)

- Some toxins can even be harmful in more than one way, such as cylindrospermopsin, which not only have the ability to harm the liver, but are also harmful to kidneys and may even cause cancer

https://www.greenwaterlab.com/what-are-algal-toxins/

Microcystis

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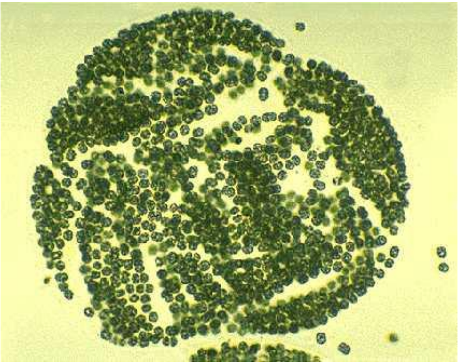
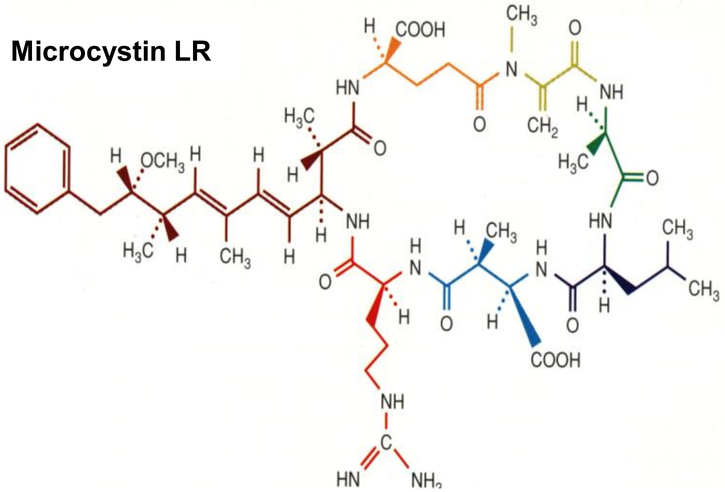


Microcystin LR



Various Microcystins

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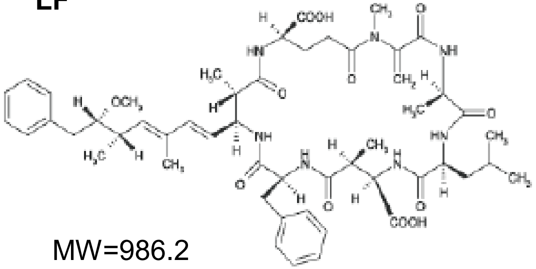
LF



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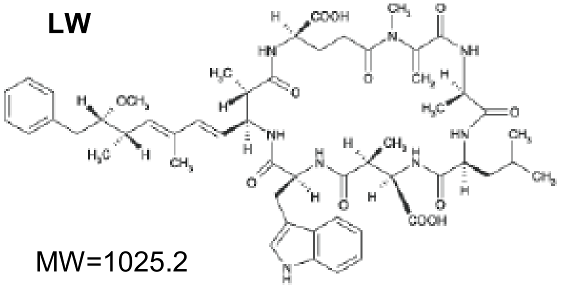
LW



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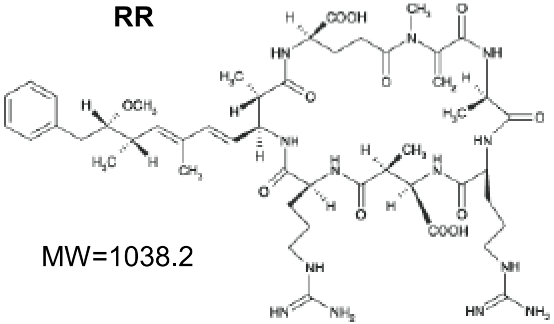
RR



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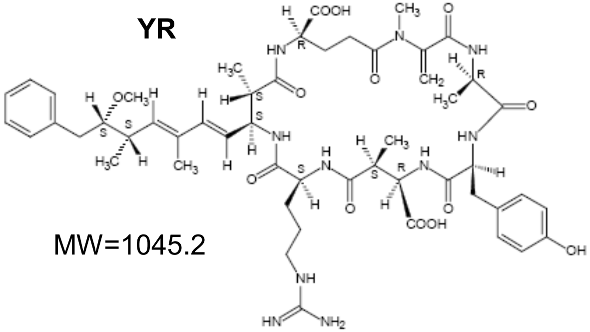
YR



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Various Microcystins and others

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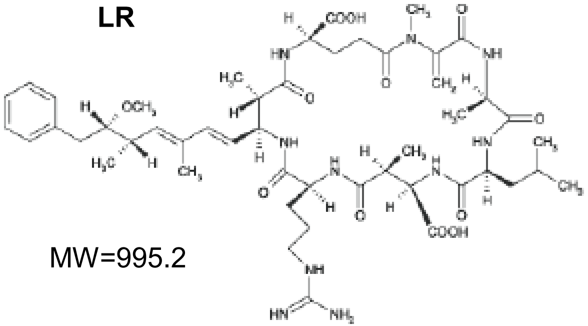
LR



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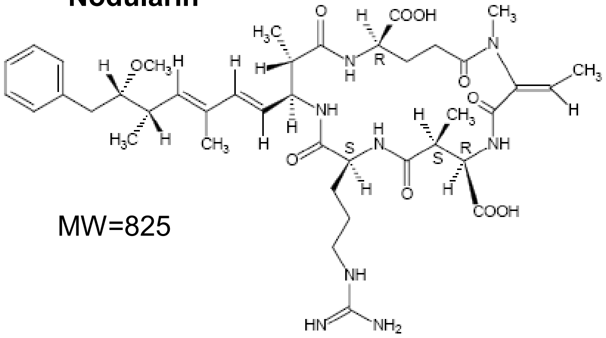
Nodularin



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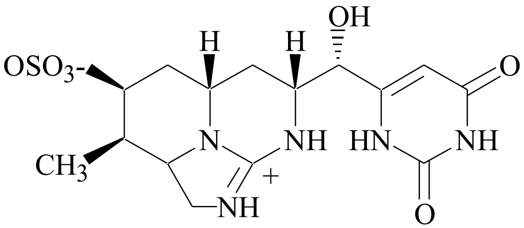
Cylindrospermopsin



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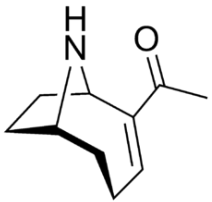
Anatoxin



MW=165.2

Microcystin Detection Assay

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- 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

System Used…UltraPerfomance LC/MS/MS

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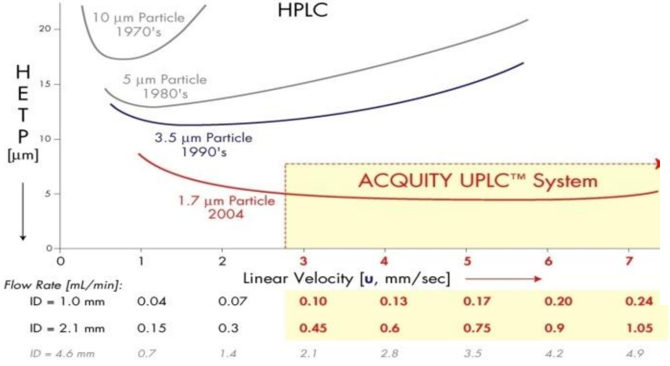
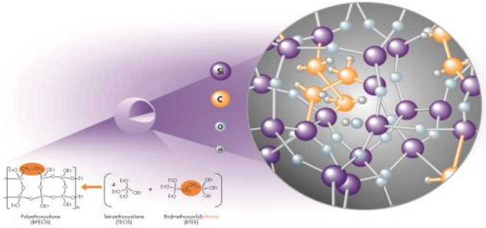
- Acquity HClass UPLC and Xevo™ TQ-S micro or TQ Absolute
- HSS T3 2.1x100mm Column (1.8um)
  - Higher separation power
  - Higher tensile strength
- Aqueous Formic/ACN Gradient





Acquity™ Ultra Performance LC

Xevo TQ Absolute

Xevo™ TQ-S micro

Tandem Experiments done by MRM

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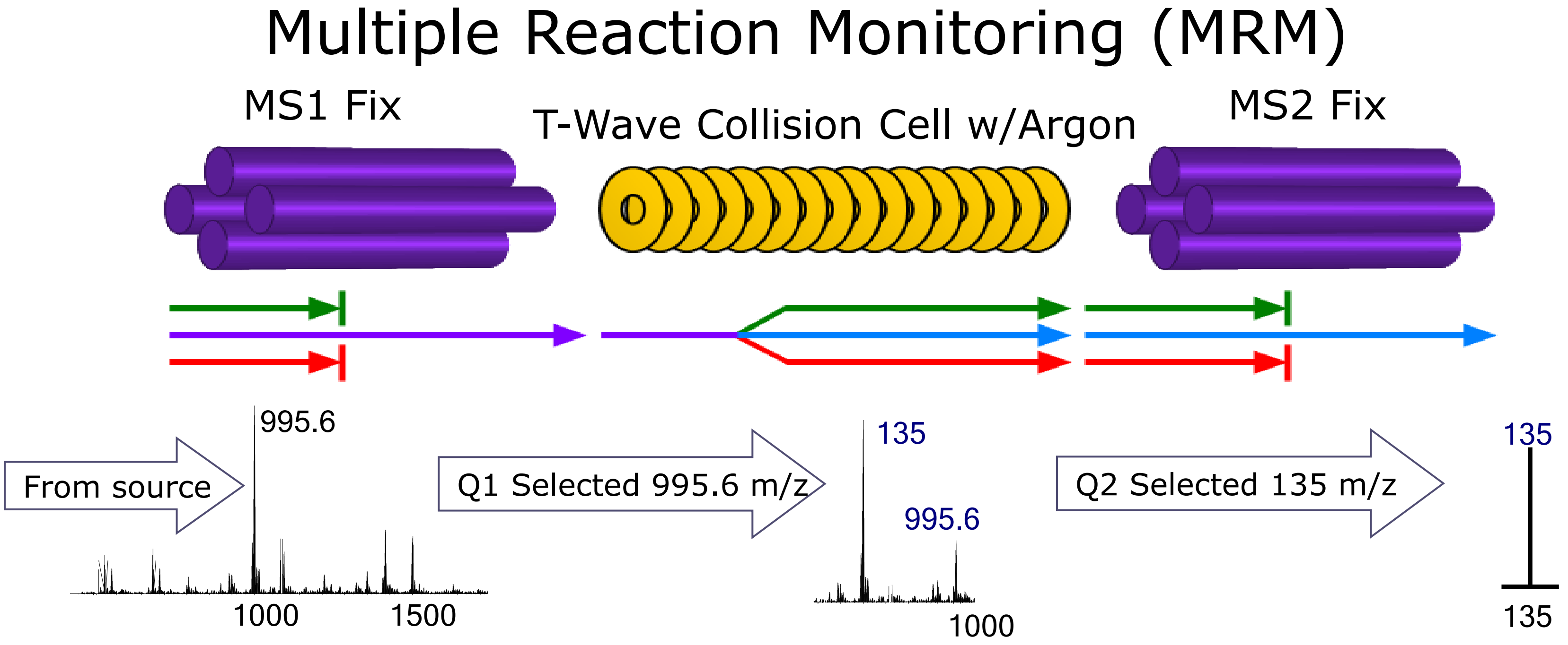


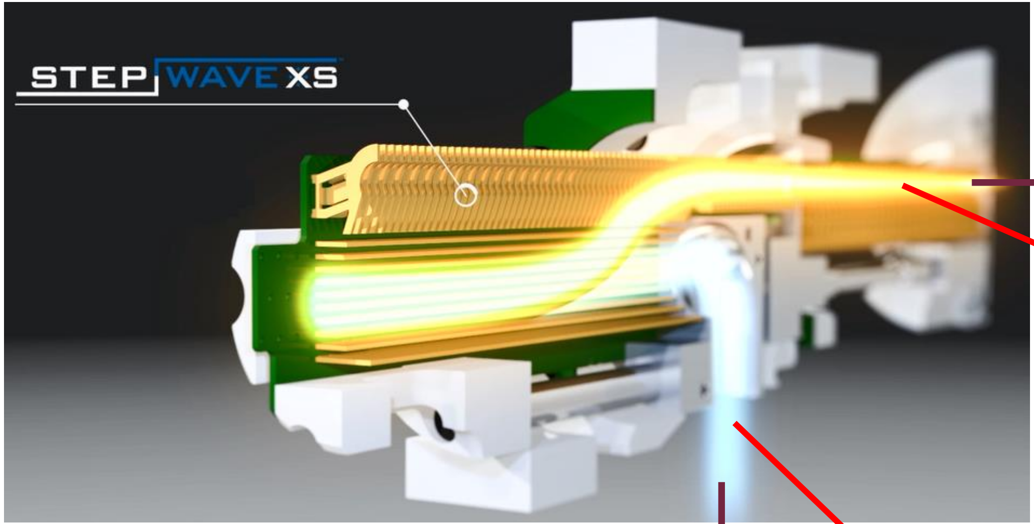
- The system is set up for selectivity, allowing only a selected product ion to be fragmented and one fragment ion to be detected.
- Multiple MRM's can also be use, as well as several fragments from a specified product ion for confirmation purposes.

Regulated Methods

- Method 544: Determination of Microcystins and Nodularin in Drinking Water
- Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water
- Method 546: Determination of Total Microcystins and Nodularins in Drinking and Ambient Waters
- ISO 22104:2021: Water quality — Determination of microcystins — Method using liquid chromatography and tandem mass spectrometry (LC-MS/MS)
- Guidelines for Canadian Recreational Water Quality - Cyanobacteria and their Toxins

STEP WAVE XS™

- Same principle as original StepWave ion guide



Ions (yellow) actively moved upwards and on-axis relative to the MS analyser

Neutrals and gas flow (blue) off-axis relative to the MS analyser and are passively removed

UPLC/MS/MS Analysis (Current Method used for Targeted Screening)

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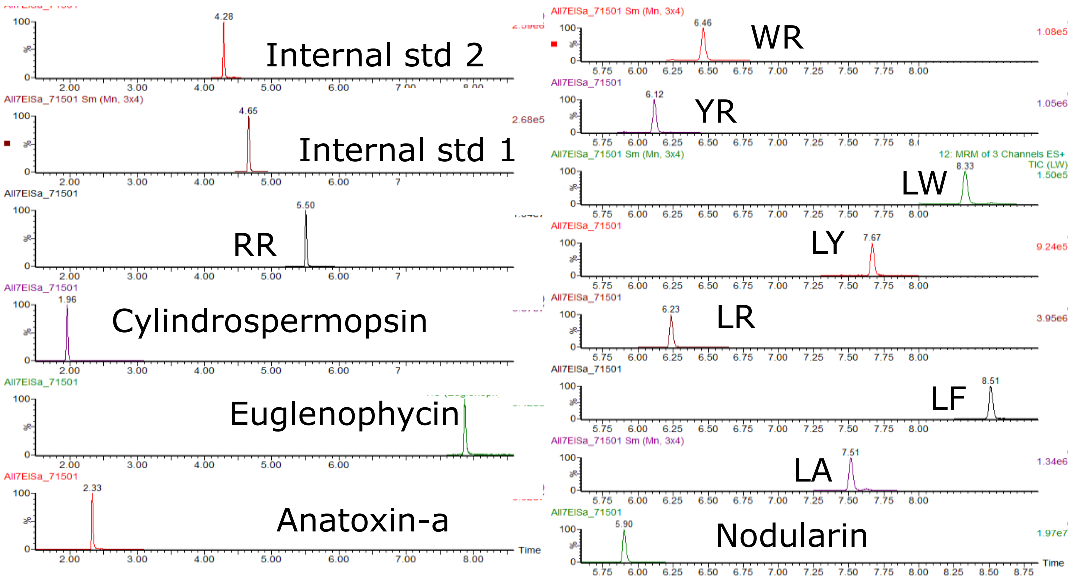
- System used.
  - H-Class UPLC and Xevo TQ-S micro or TQ Absolute mass spectrometer
  - Targeted mrm analysis
  - UPLC method used formic acid/ACN gradient based on previous work.
- Current targeted List**

LR	Phe-Ala
RR	Anabaenopeptin A
YR	Anabaenopeptin B
LA	Cylindrospermopsin (CYN)
LF	Anatoxin
LY	Homo-Anatoxin
LW	Dihydro-Anatoxin
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	Aeroginosamide B
HilR	7-epi-CYN
SPX1	GYM-B
Deoxy-CYN	Anabaenopeptin E/F
Nodularin	Euglenophycin
Debromoaplysiatoxin1 (DAT)	Lyngbyatoxin-a (LA)



**In addition, we do screen for additional toxins epoxy-anatoxin, dihydrohomoanatoxin and guanitoxin based on published mrm transitions

Proof of Concept—Direct injection (Xevo TQ Absolute)

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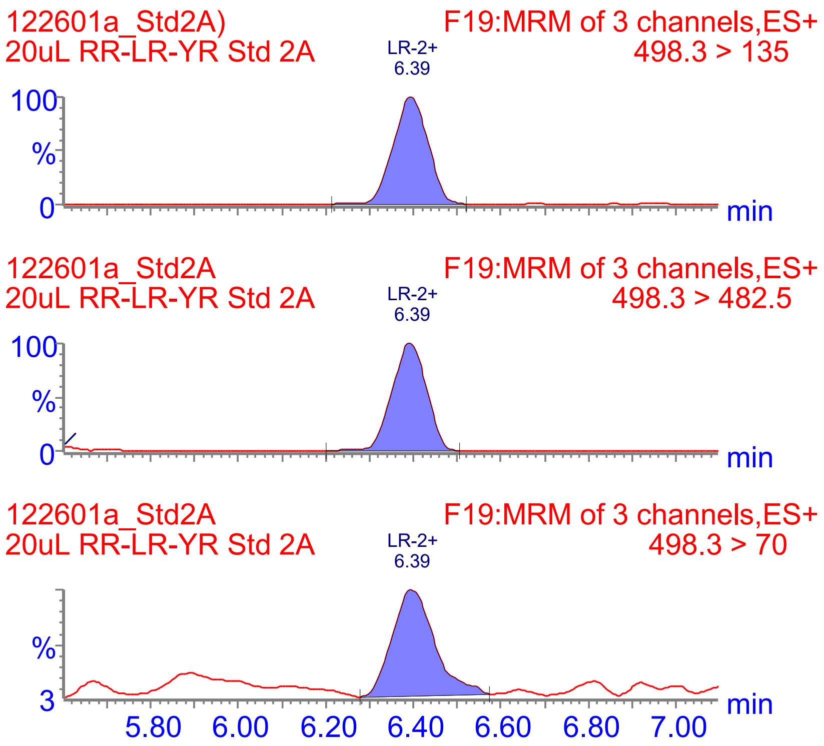
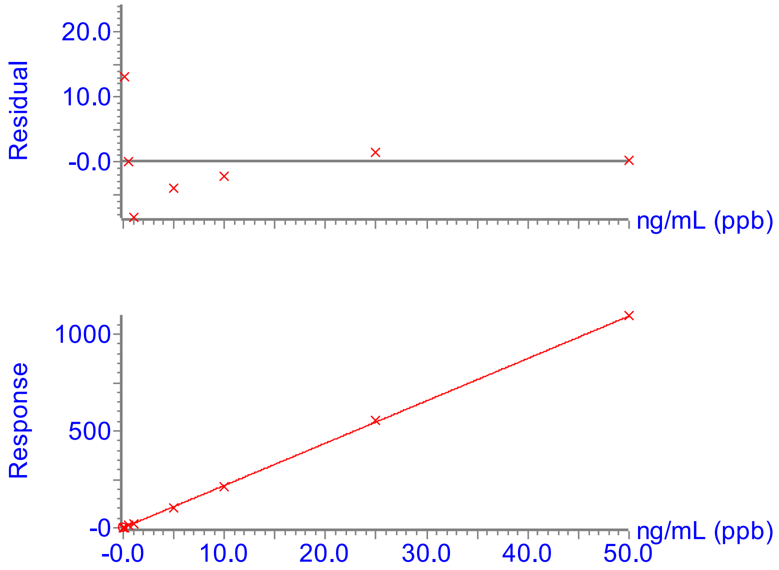
Analysis of 3 microcystins

- Certified mix of LR, RR and YR was used.
  - 20uL injection
  - 0.05ppb-50ppb calibration
- Labelled LR used as an internal standard
- Also spiked a river water sample
- Used previously developed published method (HSS T3)

LR (0.1ppb)



Compound name: LR-2+
Correlation coefficient: r = 0.999843, r^2 = 0.999687
Calibration curve: 21.8723 * x + -0.130188
Response type: Internal Std (Ref 44), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



YR (0.1ppb)

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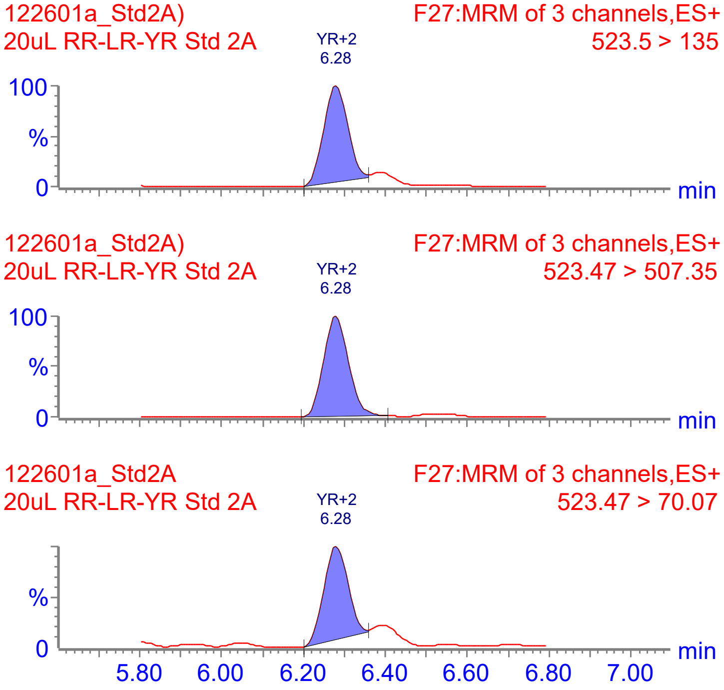
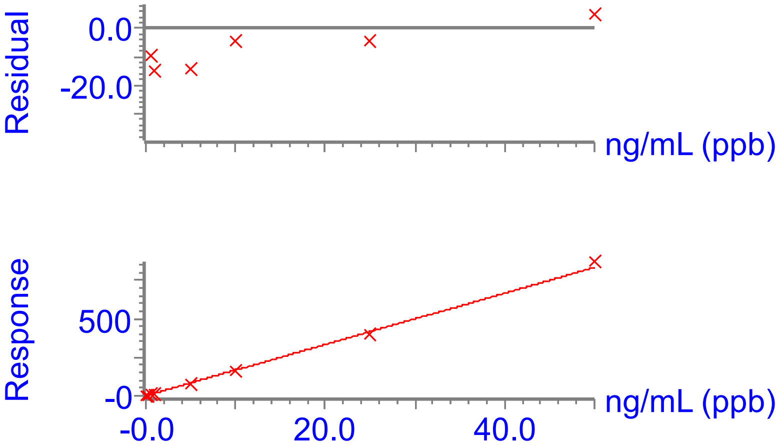
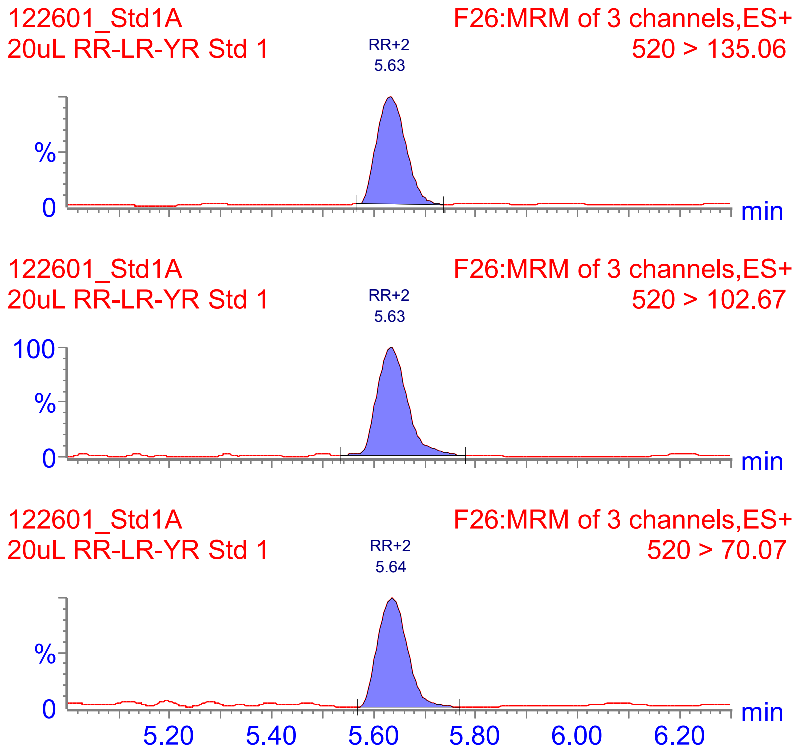


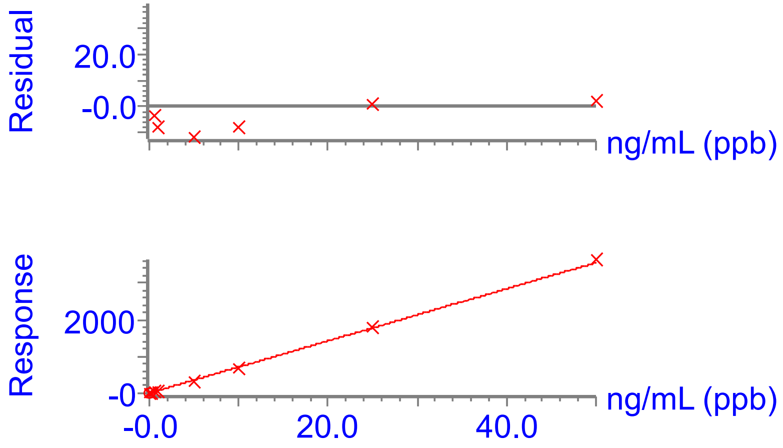
Compound name: YR
Correlation coefficient: r = 0.998031, r^2 = 0.996066
Calibration curve: 16.6485 * x + -0.172996
Response type: Internal Std (Ref 45), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



RR (0.1ppb)

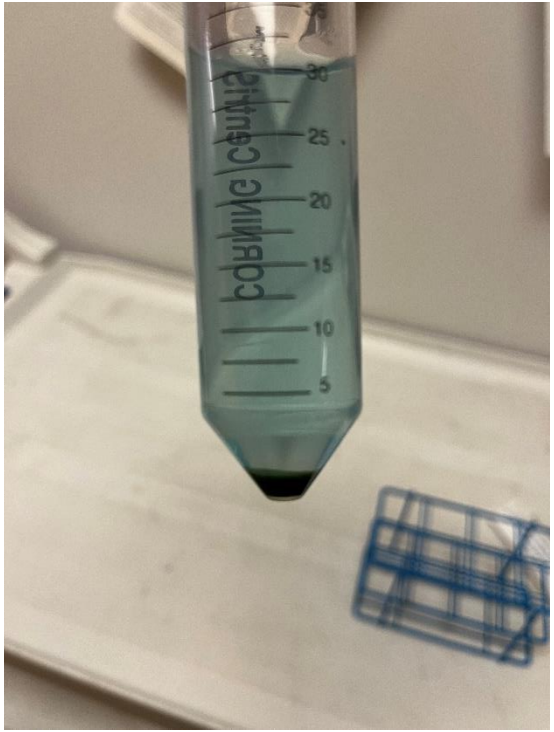


Compound name: RR+2
Correlation coefficient: r = 0.998981, r^2 = 0.997963
Calibration curve: 71.232 * x + -3.20232
Response type: Internal Std (Ref 44), Area * (IS Conc. / IS Area)
Curve type: Linear, Origin: Exclude, Weighting: 1/x, Axis trans: None



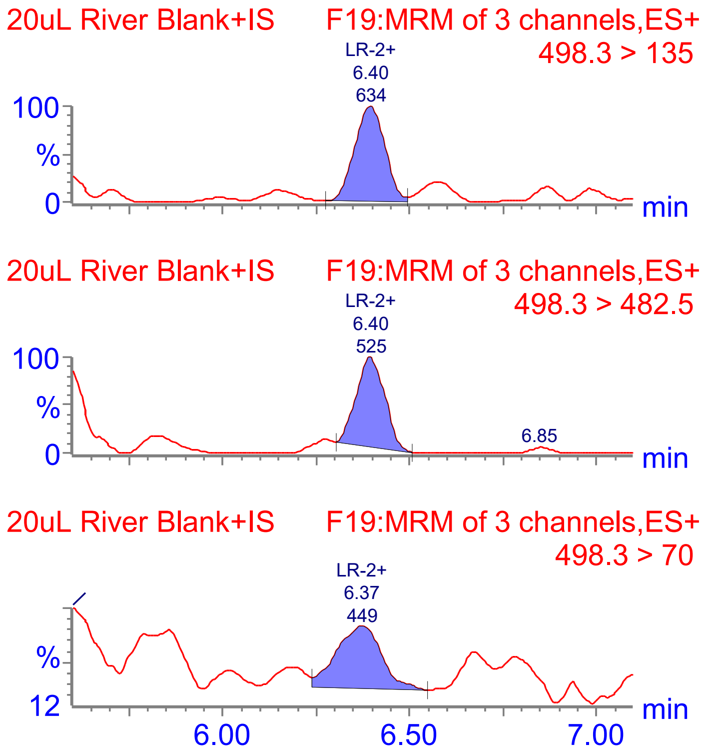
Spiked River Water

- Frozen river water samples from a bloom
  - Contained minimal toxins
  - Sample centrifuged
  - Spiked with toxins
  - Filtered and analyzed

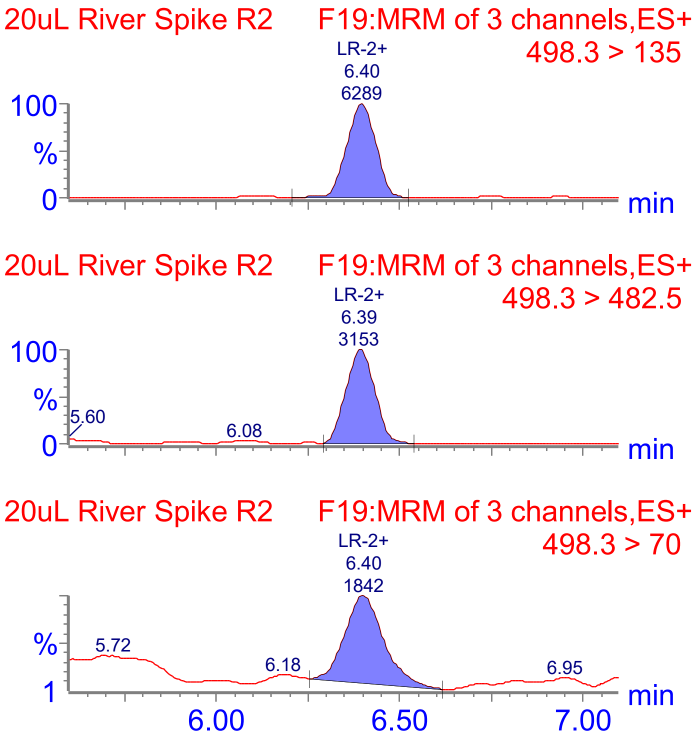


River Water

- River Water-LR (0.01ppb natural)

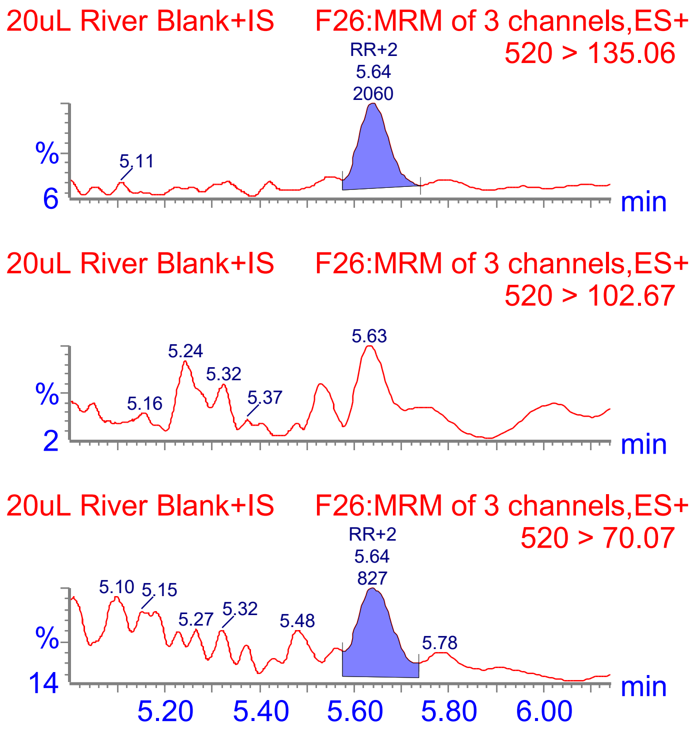


- River Water-Spiked LR (0.119ppb quantitated)

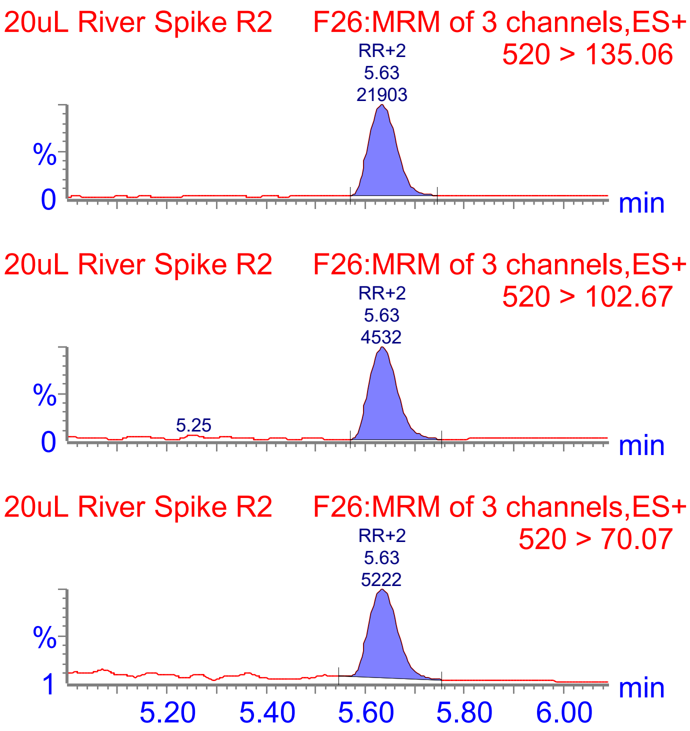


River Water

- River Water-RR (0.01ppb natural)



- River Water-Spiked RR (0.12ppb quantitated)



River Water

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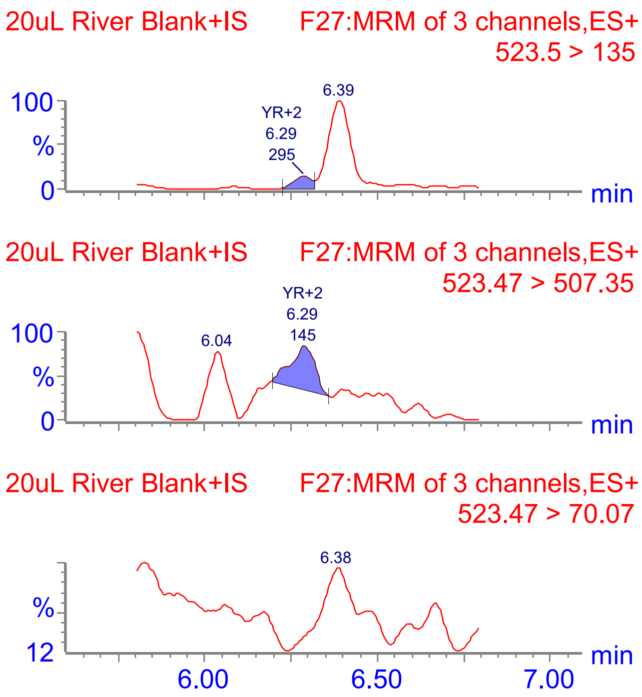
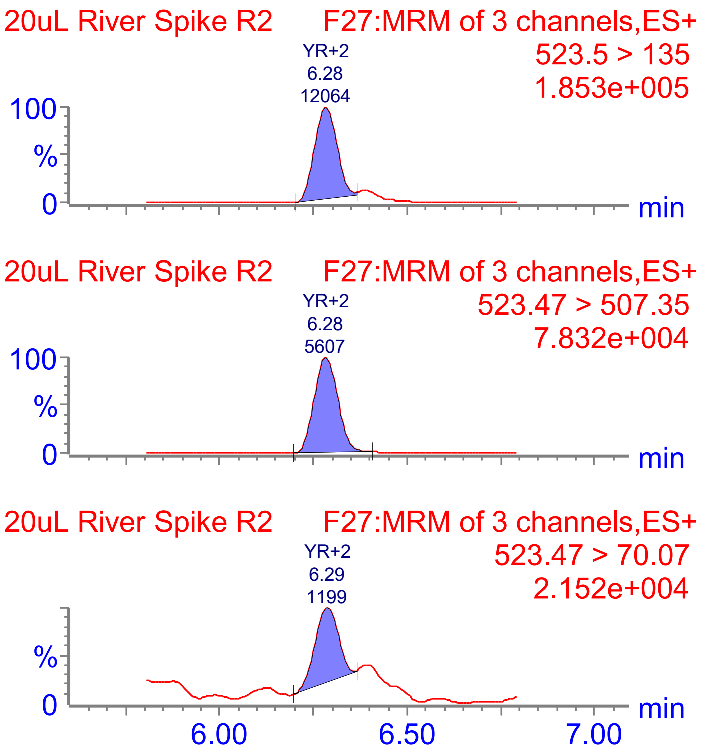
- River Water-YR (0.029ppb natural)



- River Water-Spiked YR (0.12ppb quantitated)



Spiked Results--LR

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Name	Type	Sample Text	spiked Conc (ppb)	RT	Area	Height	ng/mL (ppb)	%Dev	%Dev (with natural)
RiverBlkIS_122602A	Analyte	20uL River Blank+IS		6.4	634	6761	0.014		0
RiverR1_122601A	Analyte	20uL River Spike R1	0.05	6.39	3191	31566	0.063	26.2	-1.56
RiverR2_122601A	Analyte	20uL River Spike R2	0.1	6.4	6289	68494	0.119	19.3	4.39
RiverR3_122601A	Analyte	20uL River Spike R3	0.5	6.4	31072	331503	0.551	10.2	7.20
RiverR4_122601A	Analyte	20uL River Spike R4	1	6.4	58900	639934	1.061	6.1	4.64
RiverR5_122601A	Analyte	20uL River Spike R5	5	6.4	318208	3430839	5.519	10.4	10.07
RiverR6_122601A	Analyte	20uL River Spike R6	10	6.4	609551	6585329	11.16	11.6	11.44
122602QC_Std3A	QC	20uL RR-LR-YR Std 3	0.5	6.4	32069	347436	0.486	-2.8	
122602QC_Std4A	QC	20uL RR-LR-YR Std 4-QC	1	6.39	58746	633847	0.922	-7.8	

- % Dev (with natural) includes adding the naturally occurring toxin detected in the river sample (0.014ppb) and calculating the % Dev (with natural) from that

Spiked Results--RR

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Name	Type	Sample Text	spiked Conc (ppb)	RT	Area	Height	ng/mL (ppb)	%Dev	%Dev (with natural)
RiverBlkIS_122602A	Analyte	20uL River Blank+IS		5.64	2060	27599	0.008		0
RiverR1_122601A	Analyte	20uL River Spike R1	0.05	5.63	12478	191361	0.069	37.7	18.97
RiverR2_122601A	Analyte	20uL River Spike R2	0.1	5.63	21903	338772	0.122	21.6	12.96
RiverR3_122601A	Analyte	20uL River Spike R3	0.5	5.63	104688	1627824	0.566	13.1	11.42
RiverR4_122601A	Analyte	20uL River Spike R4	1	5.63	198222	3074308	1.094	9.4	8.53
RiverR5_122601A	Analyte	20uL River Spike R5	5	5.63	1061689	16519510	5.667	13.3	13.16
RiverR6_122601A	Analyte	20uL River Spike R6	10	5.63	2070205	32190990	11.672	16.7	16.63
122602QC_Std3A	QC	20uL RR-LR-YR Std 3	0.5	5.63	94100	1469767	0.434	-13.2	
122602QC_Std4A	QC	20uL RR-LR-YR Std 4-QC	1	5.63	177856	2758669	0.855	-14.5	

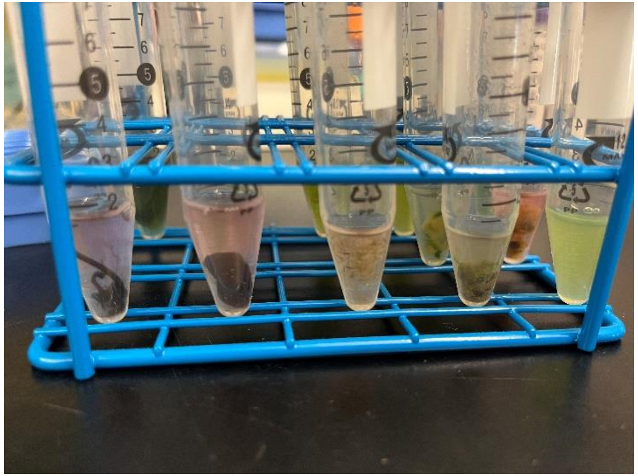
- % Dev (with natural) includes aiding the naturally occurring toxin detected in the river sample (0.008ppb) and calculating the % Dev (with natural) from that

Spiked Results--YR

Name	Type	Sample Text	spiked Conc (ppb)	RT	Area	Height	ng/mL (ppb)	%Dev	%Dev (with natural)
RiverBlkIS_122602A	Analyte	20uL River Blank+IS		6.29	295	4930	0.029		0
RiverR1_122601A	Analyte	20uL River Spike R1	0.05	6.28	5317	81379	0.07	39.6	-11.39
RiverR2_122601A	Analyte	20uL River Spike R2	0.1	6.28	12064	177501	0.124	23.9	-3.88
RiverR3_122601A	Analyte	20uL River Spike R3	0.5	6.28	63902	904272	0.524	4.8	-0.95
RiverR4_122601A	Analyte	20uL River Spike R4	1	6.28	122519	1736651	1.001	0.1	-2.72
RiverR5_122601A	Analyte	20uL River Spike R5	5	6.28	690899	9732097	5.333	6.7	6.04
RiverR6_122601A	Analyte	20uL River Spike R6	10	6.28	1340940	18843860	10.903	9	8.71
122602QC_Std3A	QC	20uL RR-LR-YR Std 3	0.5	6.28	70211	995183	0.493	-1.3	
122602QC_Std4A	QC	20uL RR-LR-YR Std 4-QC	1	6.28	136092	1917780	0.968	-3.2	

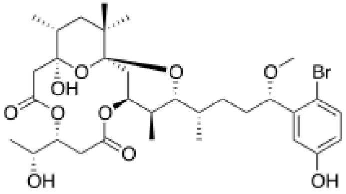
- % Dev (with natural) includes aiding the naturally occurring toxin detected in the river sample (0.029ppb) and calculating the % Dev (with natural) from that

Other “strange” things

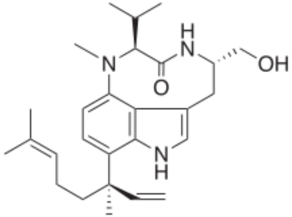


Additional Toxins?   Dermatoxins

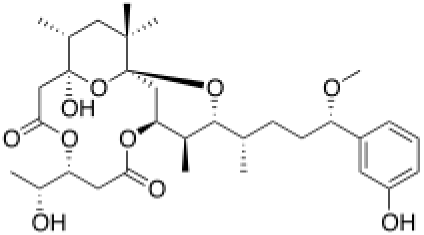
- Dermatoxins (toxins that damage the skin)
- Examples.
  - Aplysiatoxin-1 (AT)



  - Lyngbyatoxin-a

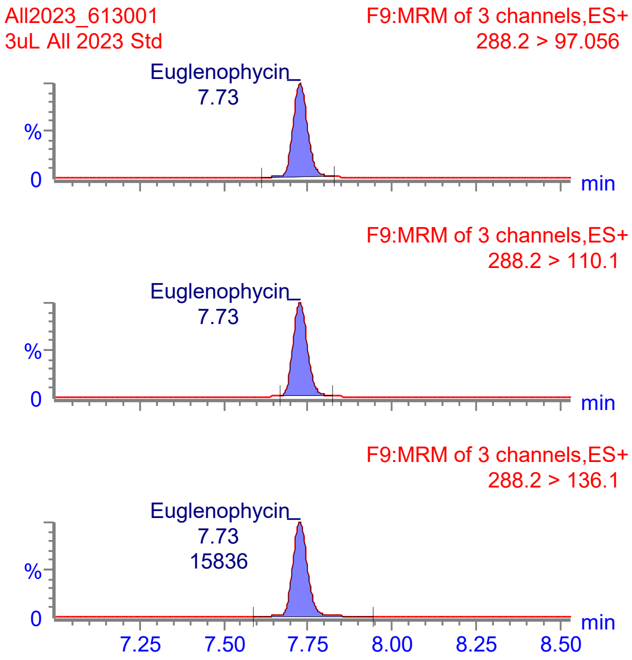


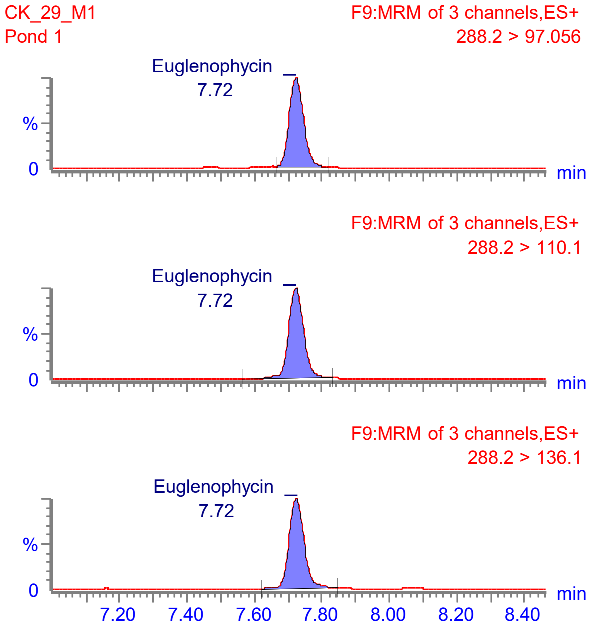
- Debromoaplysiatoxin (DAT)

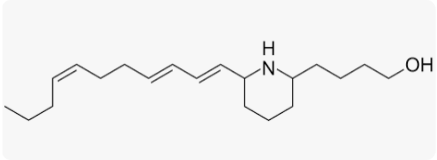


Mat samples

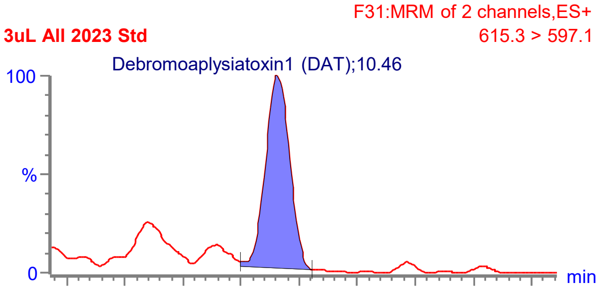
- Additional toxin found (Euglenophycin)

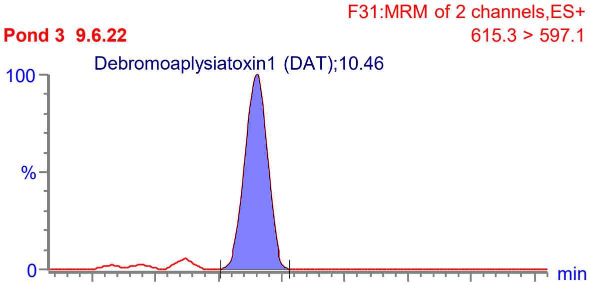


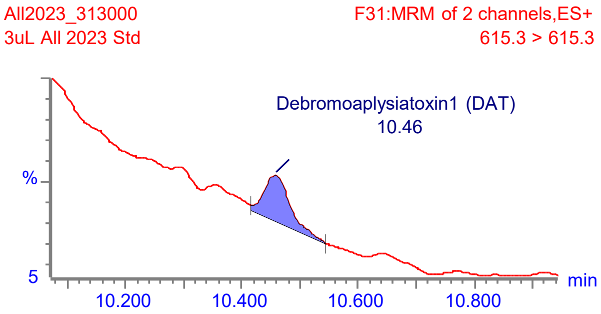


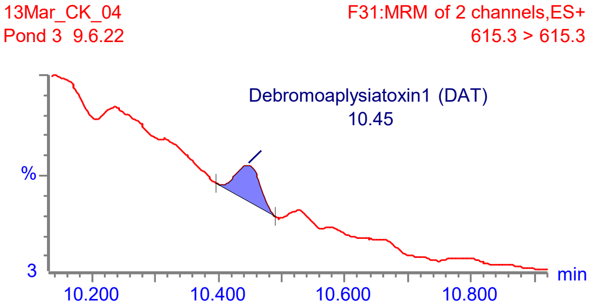


Additional Toxins—Dermatoxins (DAT)

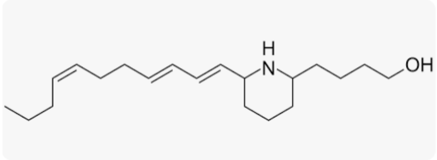


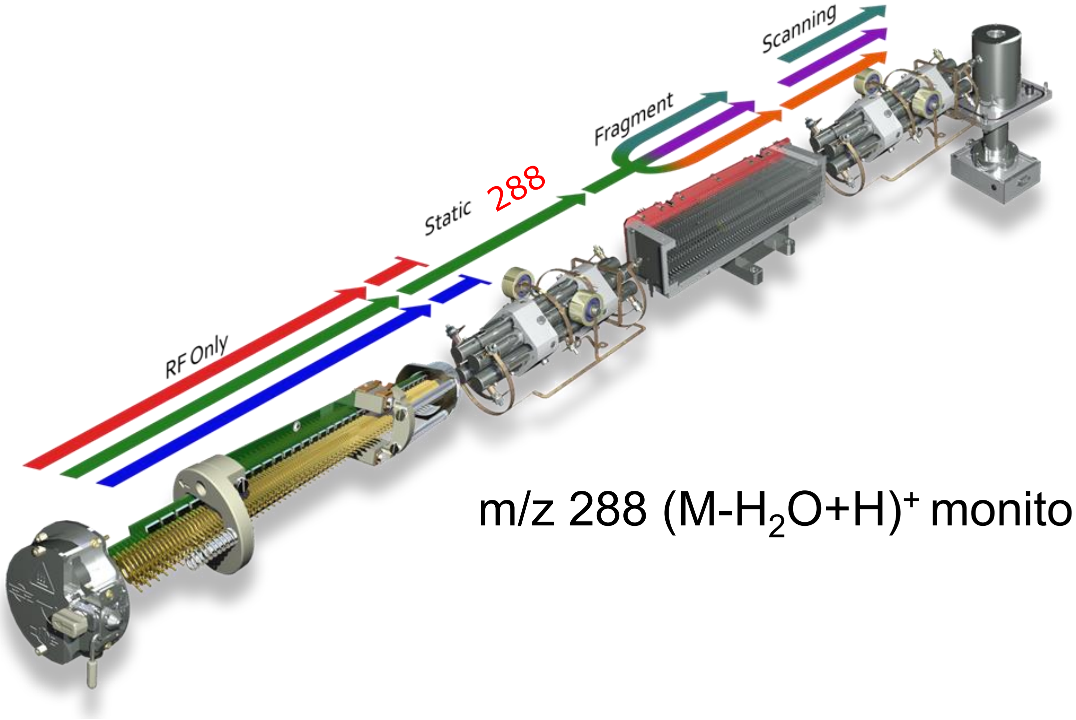






Product Ion Scanning





m/z 288 $(M-H_2O+H)^+$ monitored and fragmented

Confirmed fragment ions for Euglenophycin

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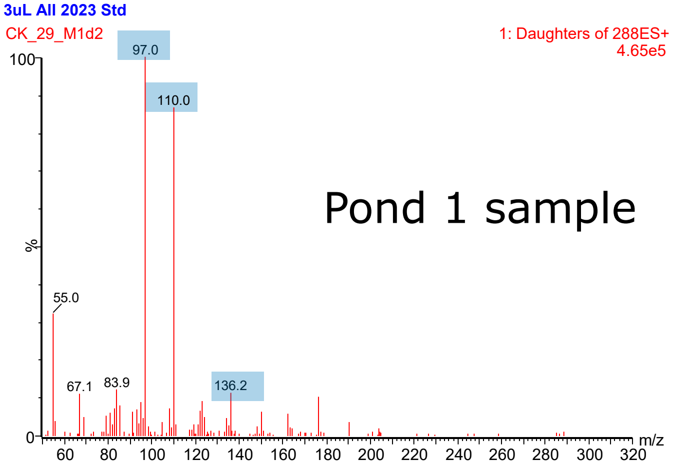
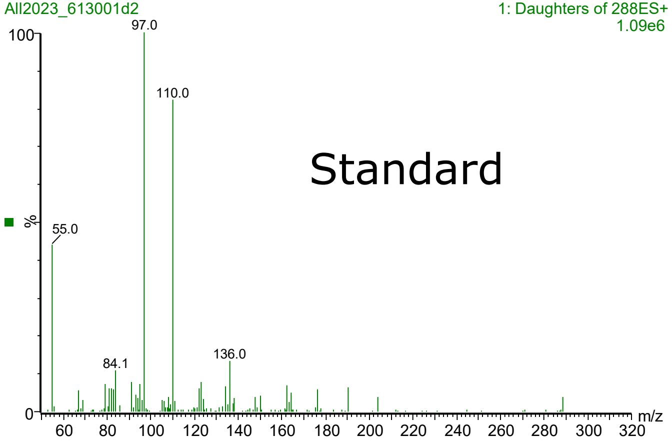
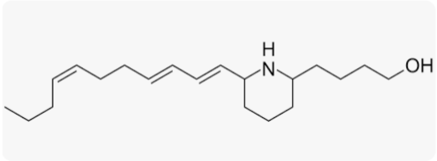




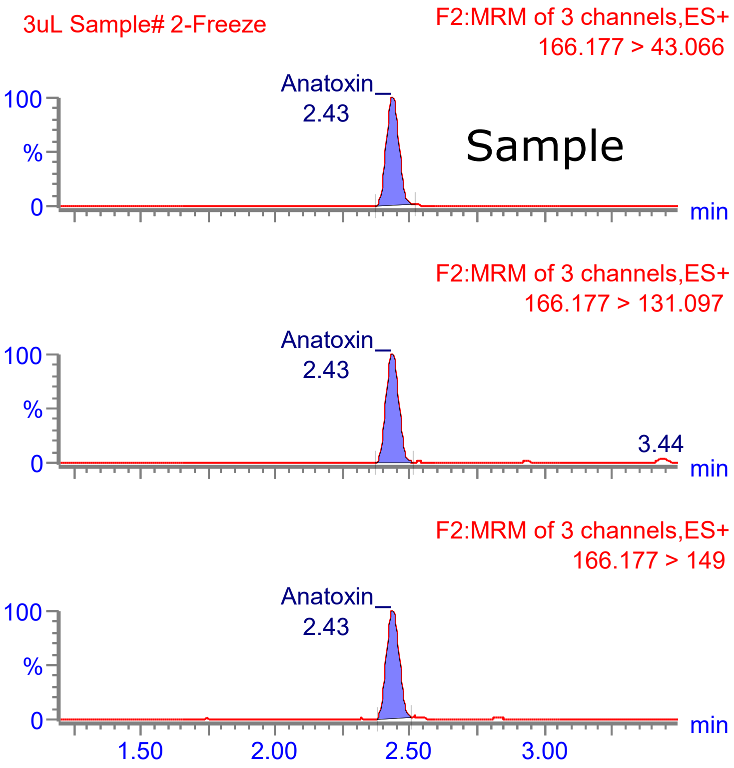


- Same fragment ions for both
- Match literature references for Euglenophycin

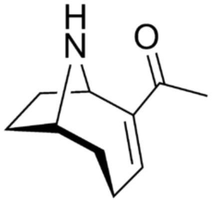
Paul V. Zimba, I-Shuo Huang, Danielle Gutierrez, Woongghi Shin, Matthew S. Bennett, Richard E. Triemer, Harmful Algae,Volume 63, 2017,Pages 79-84, ISSN 1568-9883, https://doi.org/10.1016/j.hal.2017.01.010

Water Reservoir

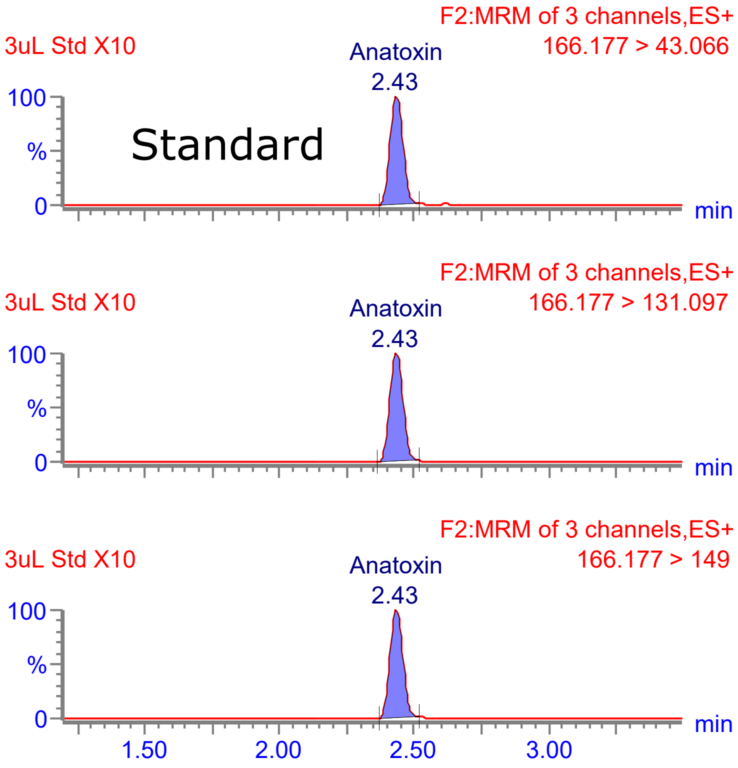
Waters™



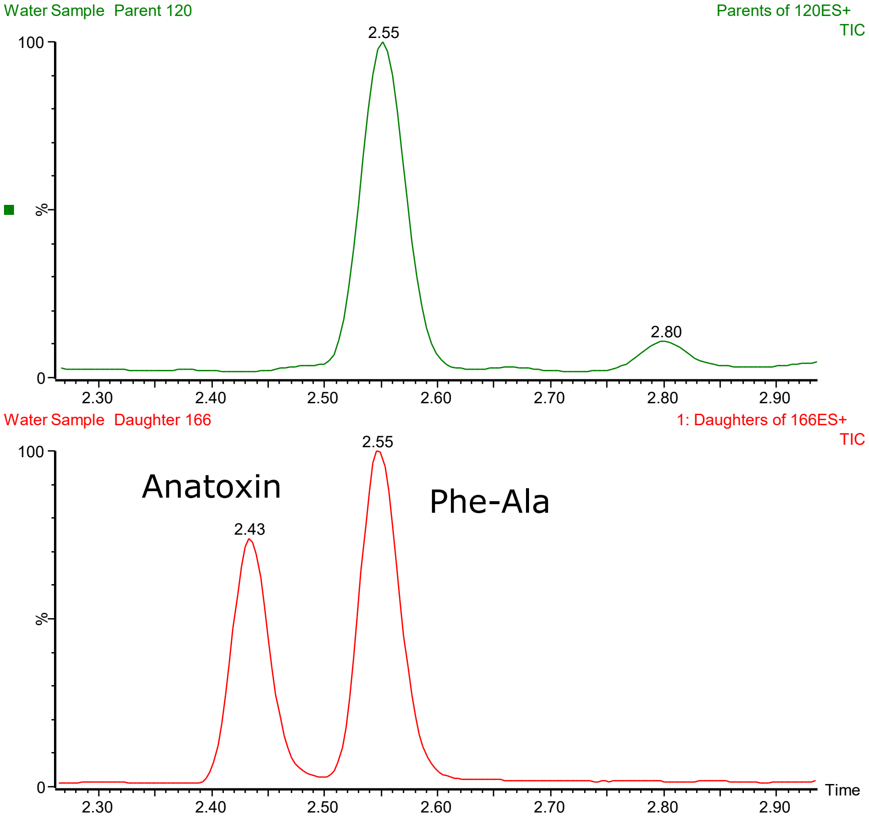
Anatoxin

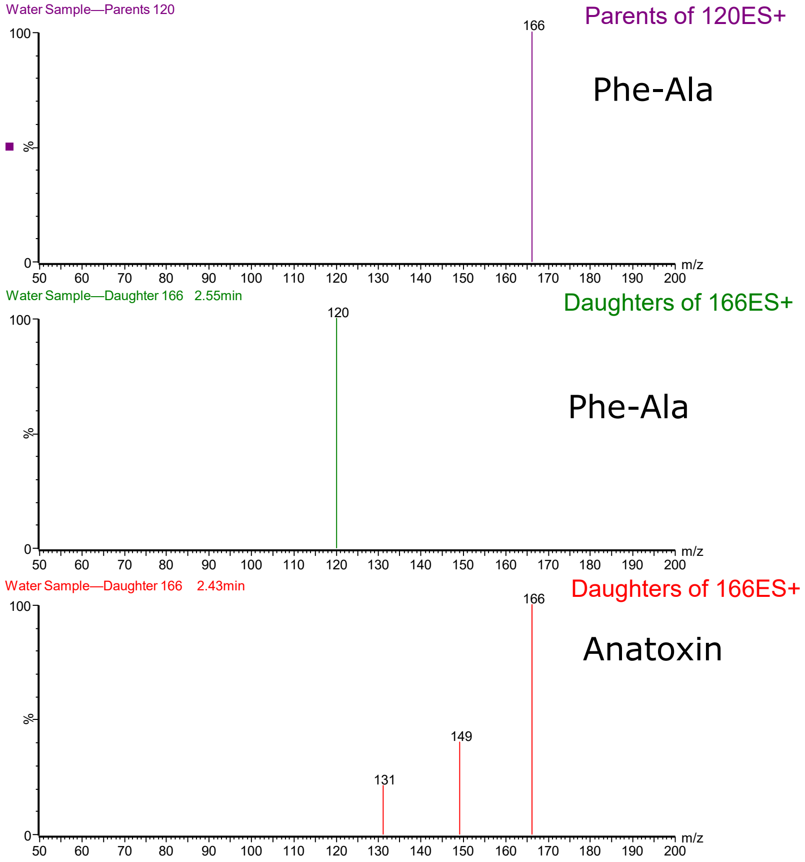


MW=165.2

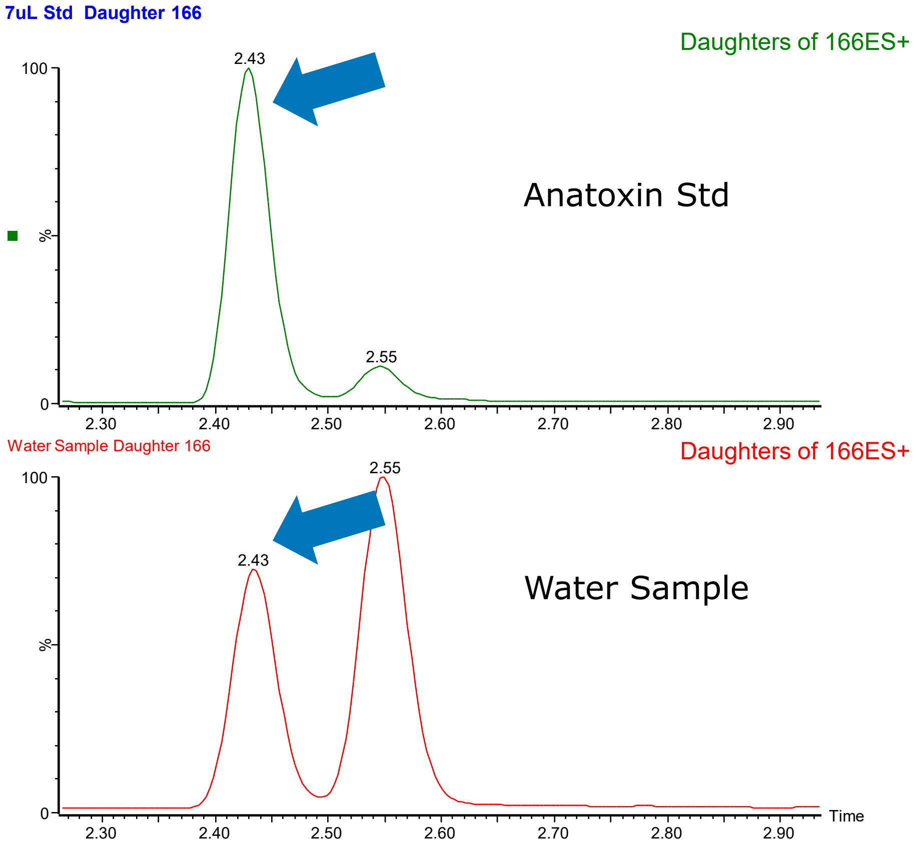


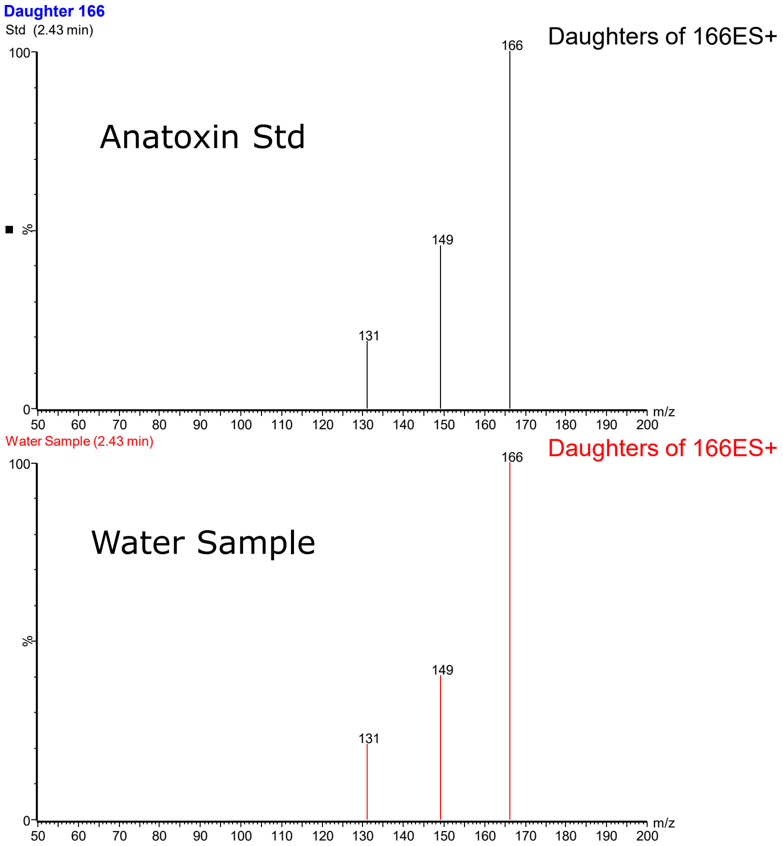
Precursor (Parents) and Product (Daughter) scanning





Comparing to known Anatoxin standard

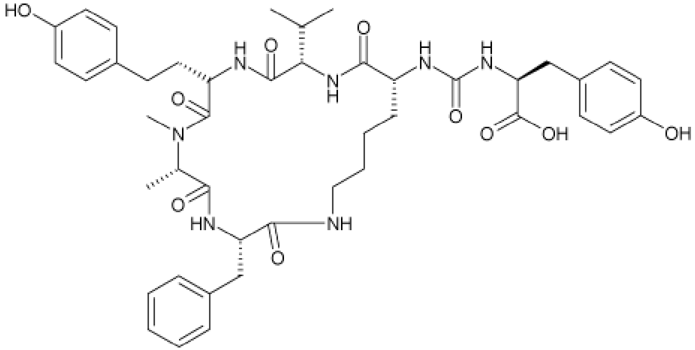




Anabaenopeptins

- 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.

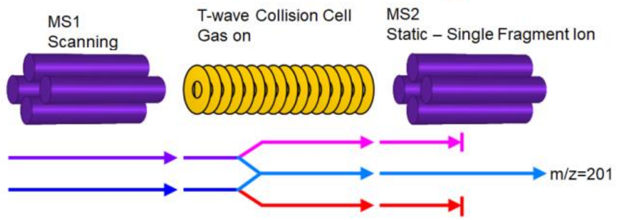
- Anabaenopeptin A



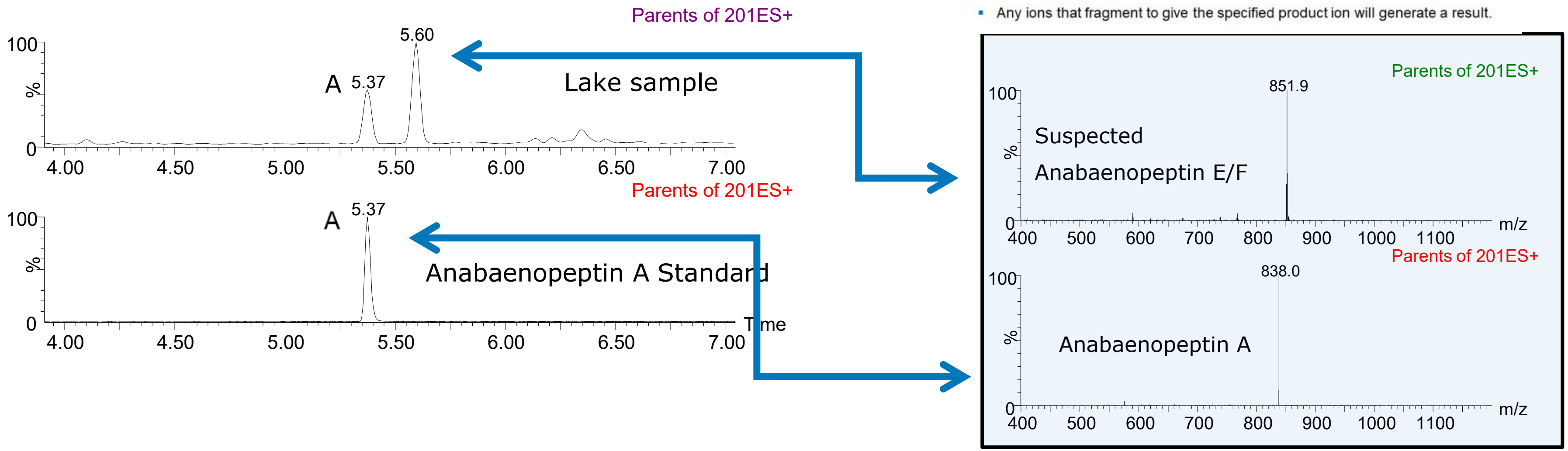
Finding more

- Looking for other anabaenopeptins
  - A and B are available as standards.
  - Common fragment ion is m/z 201
    - $(CO\text{-}Arg)^{+1}$
  - Can scan for precursor (parent) mass

Precursor (Parent) Ion Scanning



- MS1 is scanned over a specified mass range and all ions are sequentially passed through to the collision cell where they are fragmented
- MS2 is set to transmit only the mass of a specific fragment ion (i.e. m/z=201) and does not scan
- Any ions that fragment to give the specified product ion will generate a result.



Summary

Waters™

- Mass Spectrometry offers a sensitive and selective method to detect various toxins.
- UPLC offers high resolution and fast analysis times with additional toxin analysis easily added to the current method
- MS/MS technology can also be used for other critical/emerging water assays (pesticides and Persistent organic pollutants (POP's) for example).
- Further work being done using untargeted TOF instrument

Acknowledgements

Waters™

- Waters Corporation
- Northern Kentucky University

GOT TOXINS?

Questions? Interesting samples?

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