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CYANOBACTERIAL TOXINS IN RECREATIONAL WATERS USING A TARGETED UPLC/MS/MS METHOD AND COMPARSION TO METAGENOMICS

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Continued Development of a targeted method for toxin analysis

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- Traditional work done by HPLC…quickly moved to small particle (sub 2um)

- UPLC/MS/MS system used.
  - H-Class UPLC and Xevo™ TQ-S micro mass spectrometer
  - Targeted mrm analysis
  - UPLC method used formic acid/ACN gradient based on previous work.

- More compounds added as standards and research continues.
- Analyzing samples from a variety of locations

What is a Tandem Quadrupole MS?

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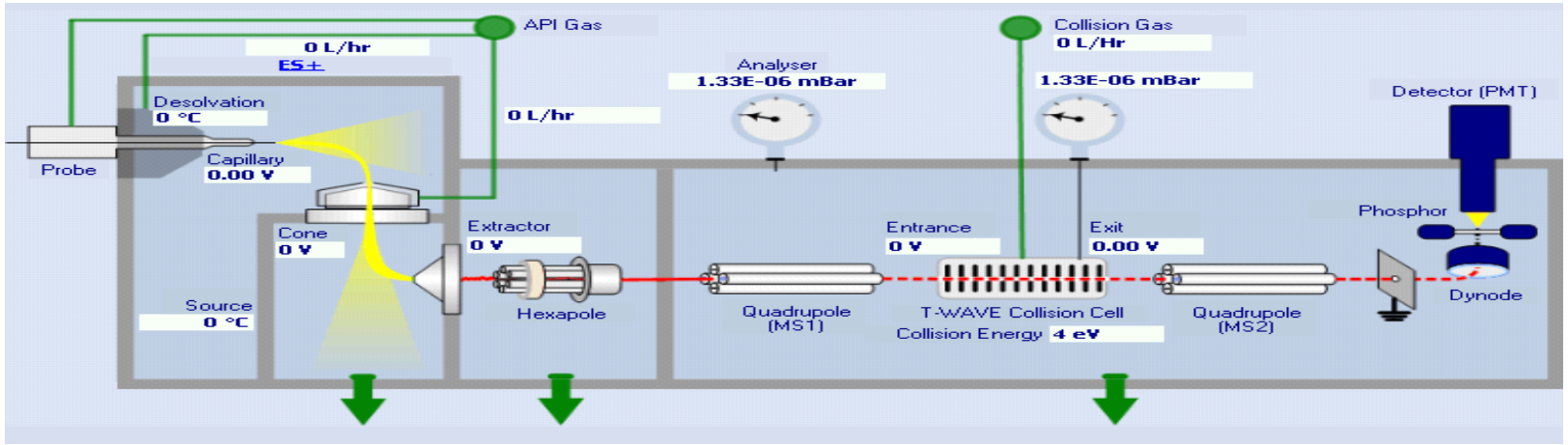
- Tandem Quadrupoles consist of 2 mass analyzers(quads),and a collision cell, enabling **more selective** analyses to be performed compared to a single quadrupole or other non MS detection mode.
- Their selectivity has made them the industry standard for MS based quantitation.
  - The ions generated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



Tandem Experiments

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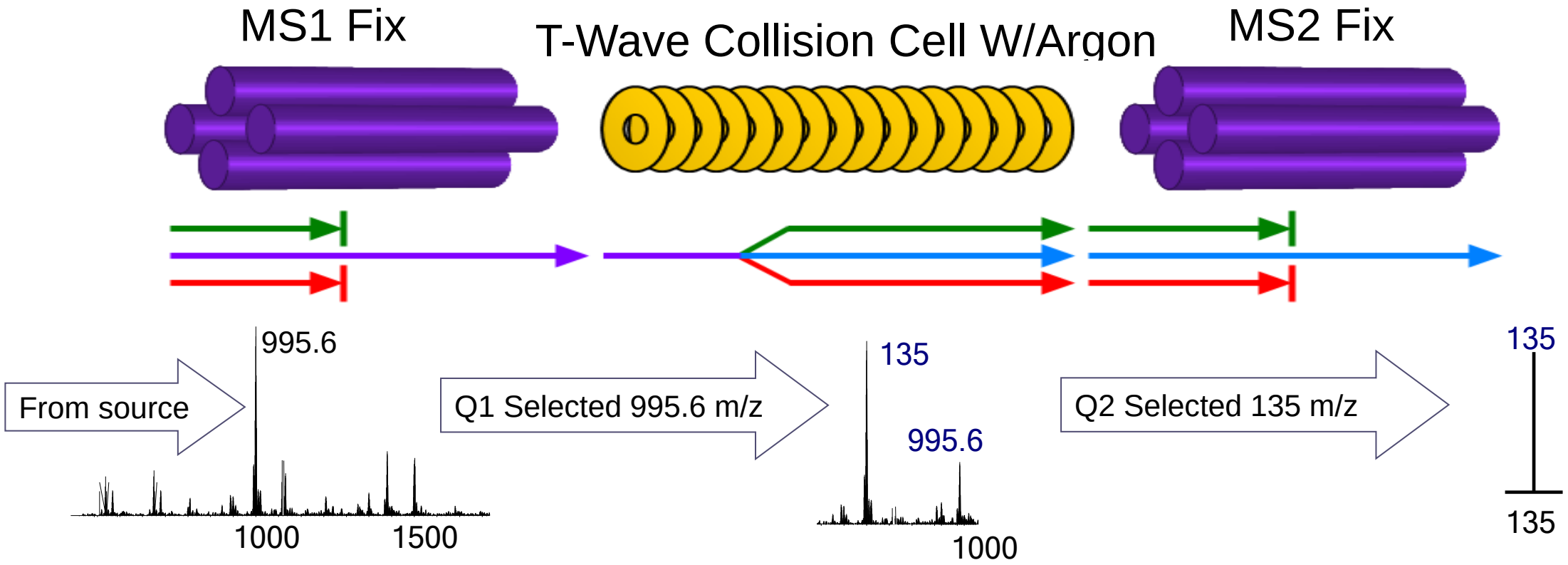
Multiple Reaction Monitoring (MRM)



- 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.

Going the Next Step…UltraPerfomance LC/MS/MS

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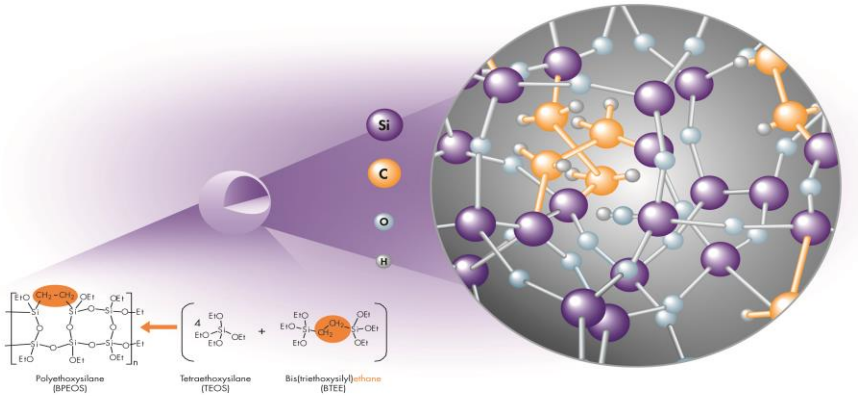
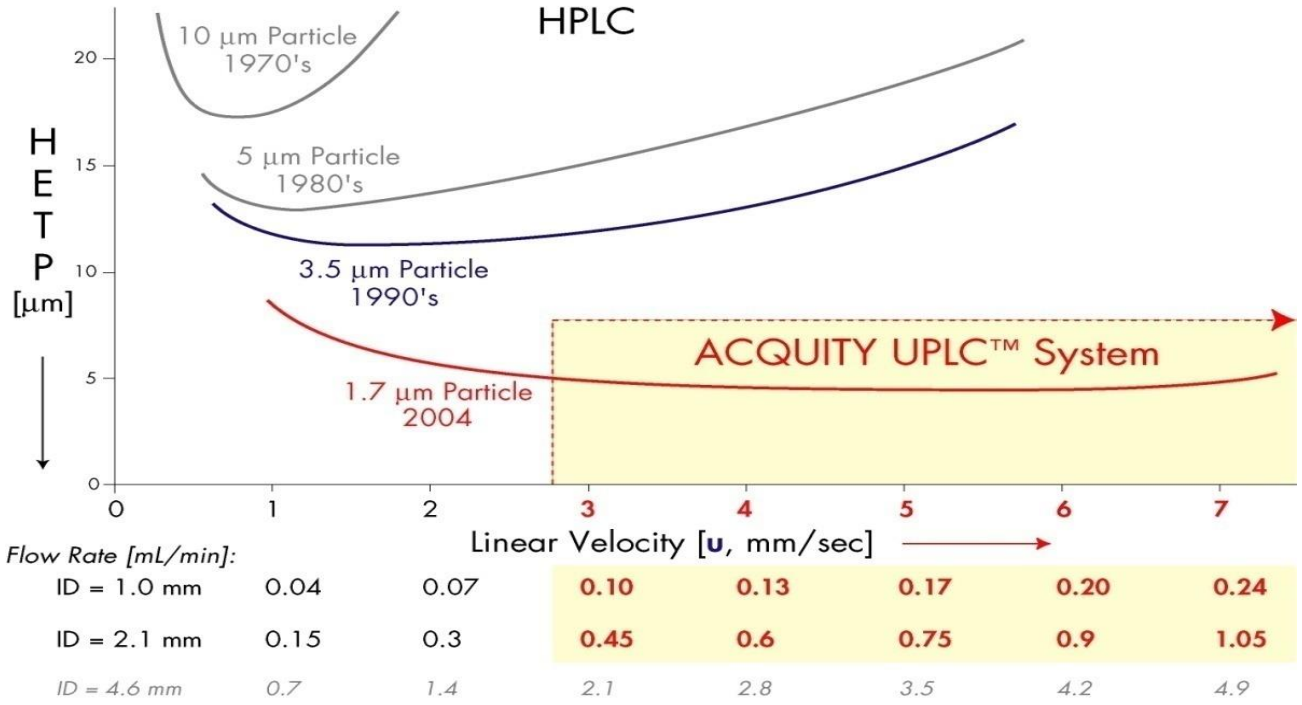
- Small Particle (sub 2µm)
- Higher separation power
- Higher tensile strength



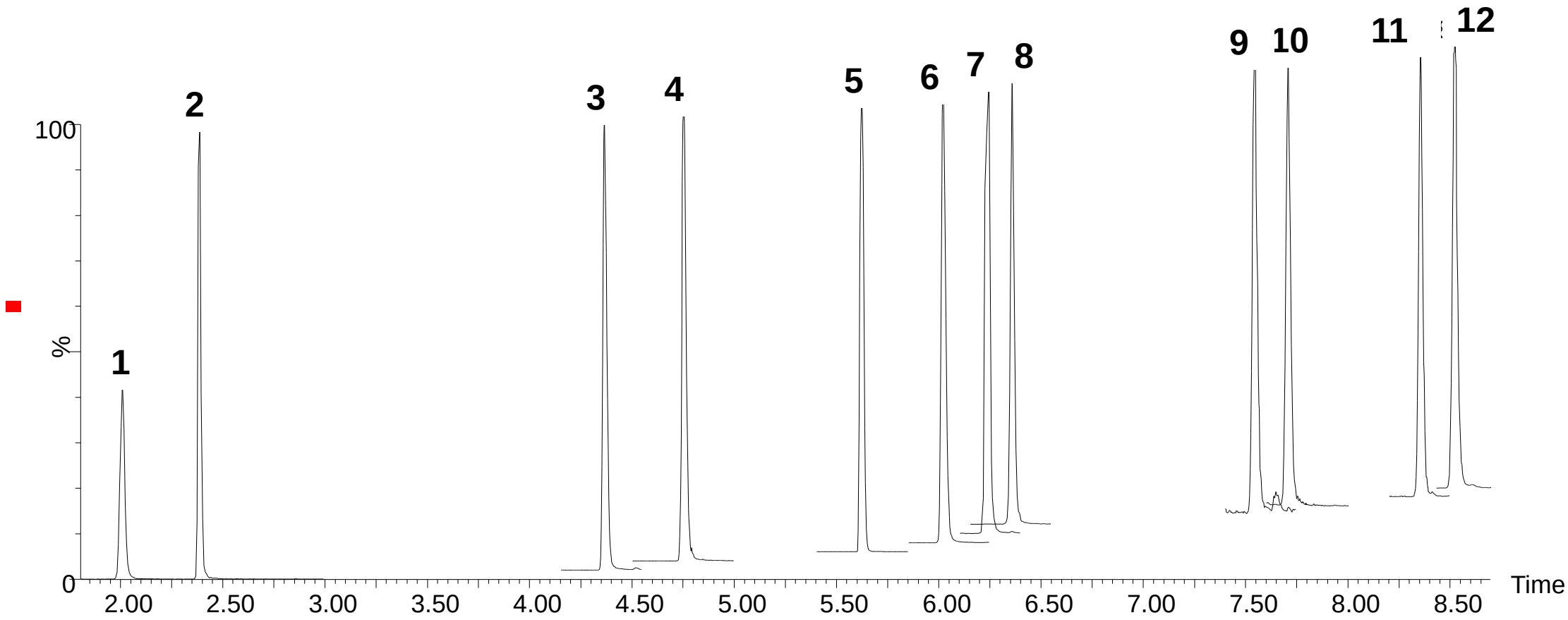


Flow Rate [mL/min]:							
ID = 1.0 mm	0.04	0.07	0.10	0.13	0.17	0.20	0.24
ID = 2.1 mm	0.15	0.3	0.45	0.6	0.75	0.9	1.05
ID = 4.6 mm	*0.7*	*1.4*	*2.1*	*2.8*	*3.5*	*4.2*	*4.9*

Xevo™ TQ-S micro

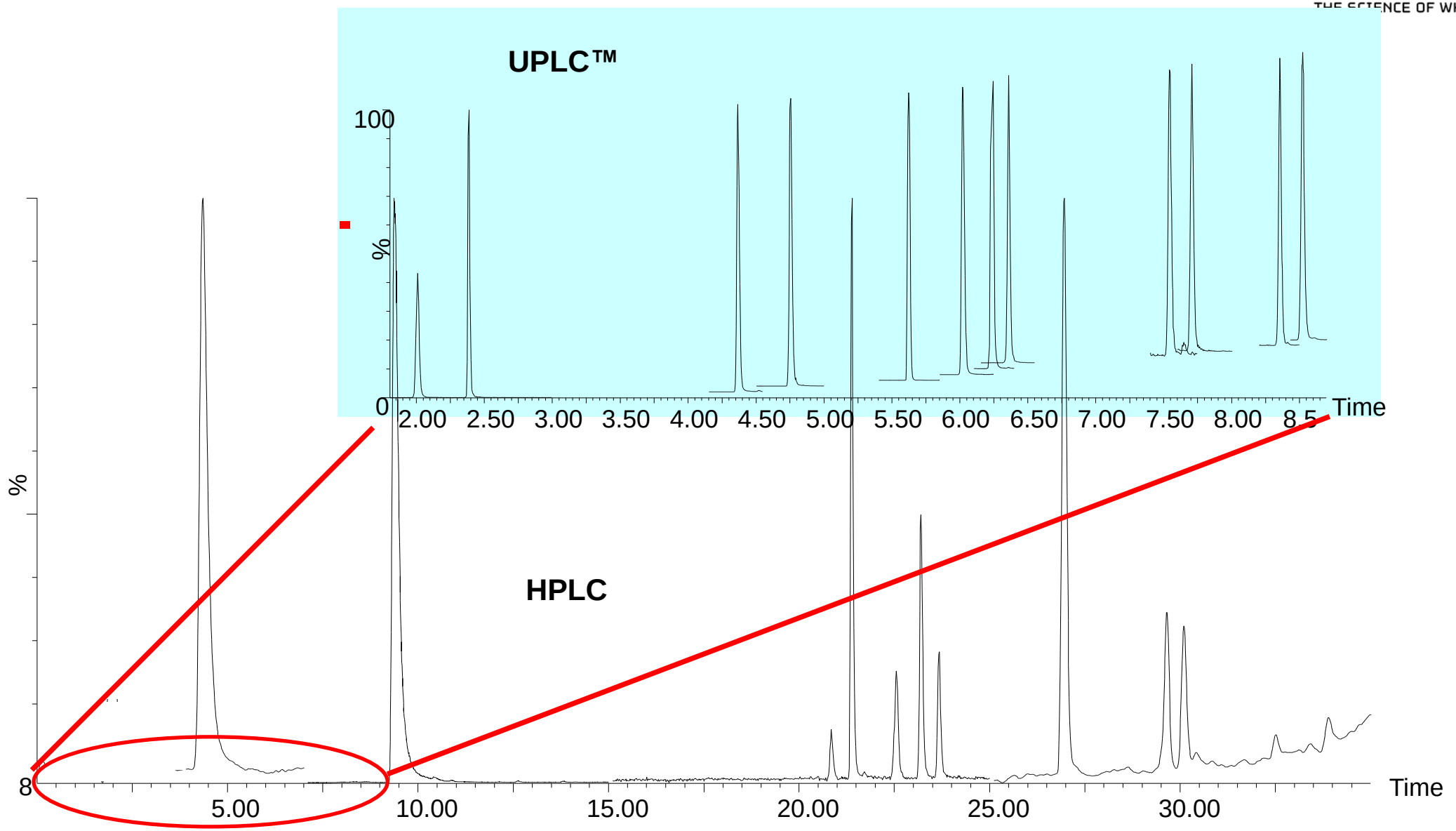
Acquity™
Ultra Performance LC

UPLCMS/MS Separation
8.5 minutes (HSS T3 2.1x100mm (1.8um))



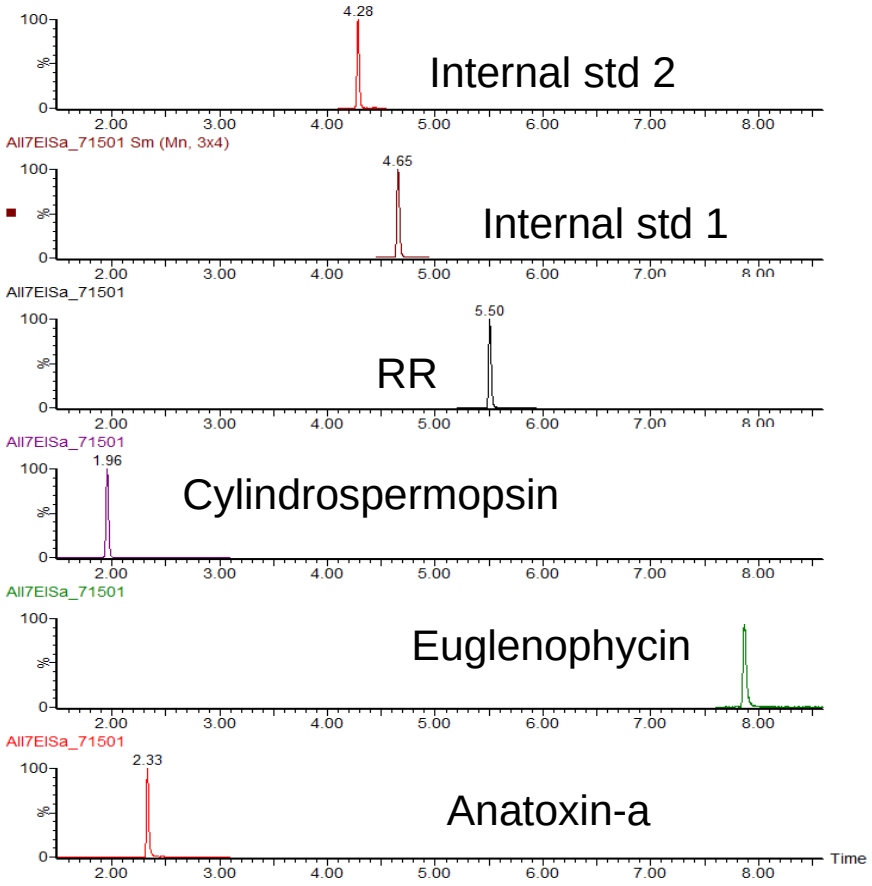
1=Cylindrospermopsin, **2**=Anatoxin-a, **3**=Cyclo (Arg-Ala-Asp-D-Phe-Val) (IStd), **4**=[Leu^5]-Enkephalin (IStd), **5**= Microcystin RR, **6**=Nodularin, **7**=Microcystin YR, **8**=Microcystin LR, **9**=Microcystin LA, **10**=Microcystin LY, **11**=Microcystin LW, and **12**=Microcystin LF

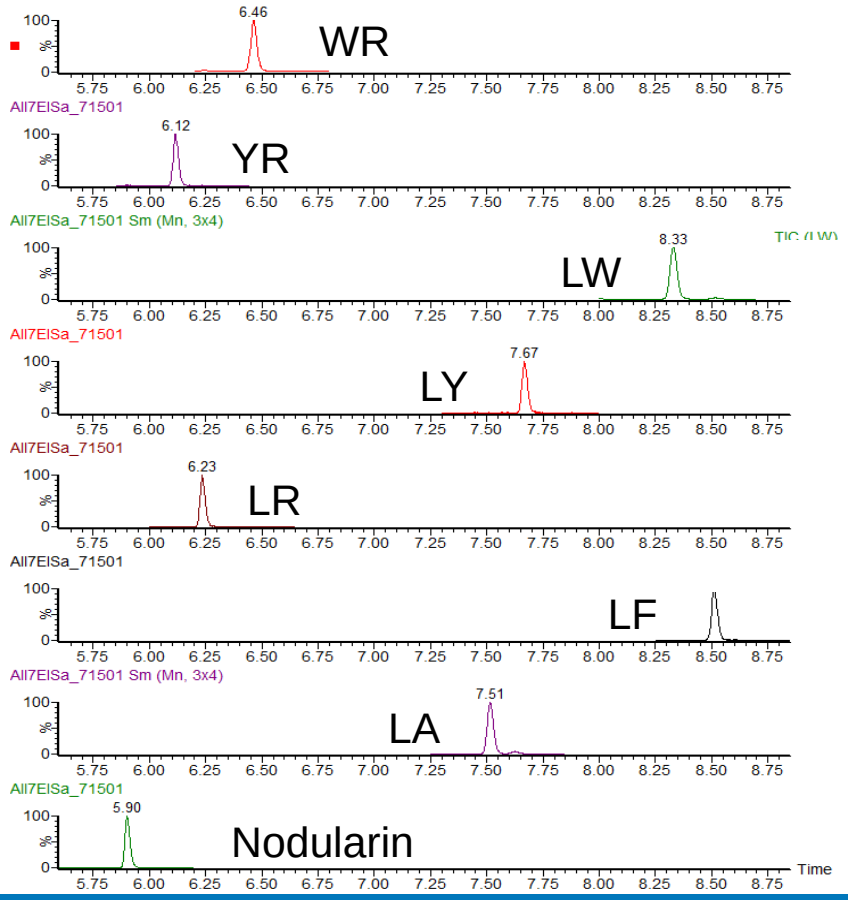
HPLC vs UPLC



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

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



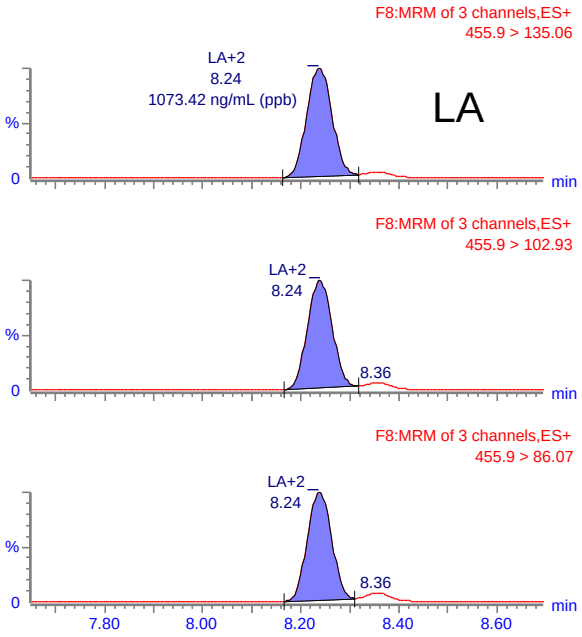


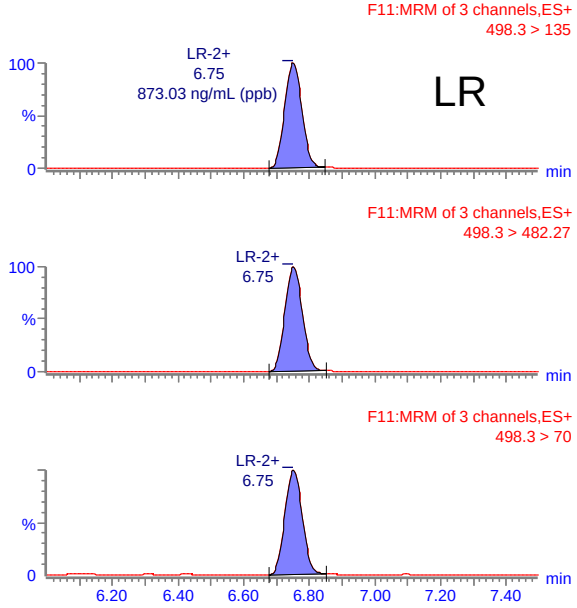
Expanded List

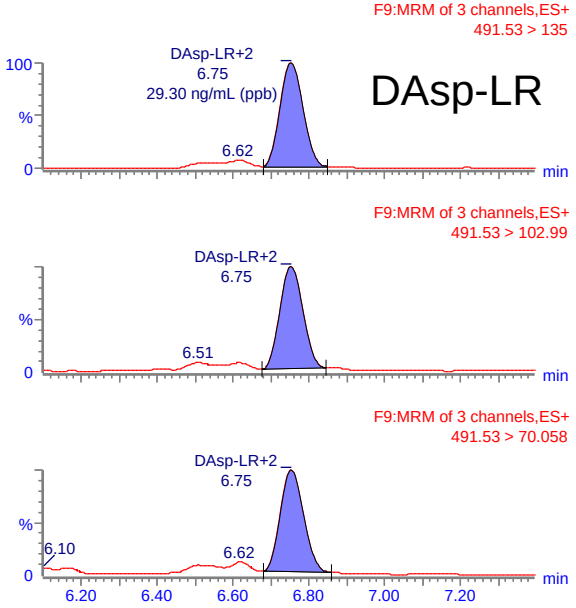
LR	Phe-Ala
RR	Anabaenopeptin A
YR	Anabaenopeptin B
LA	Cylindrospermopsin (CYN)
LF	Anatoxin
LY	Nodularin
LW	Euglenophycin
WR	Ethylated MC-LR (d5)(IS)
HtYR	PI-Cylco (IS)
D-Asp3-RR	Leu-Enk (IS)
D-Asp3-LR	Micropeptin 1106
D-Asp3-Dhb7-MC-Htyr	Aeroginosamide B
Homo-Anatoxin	7-epi-CYN
Deoxy-CYN	Anabaenopeptin E/F

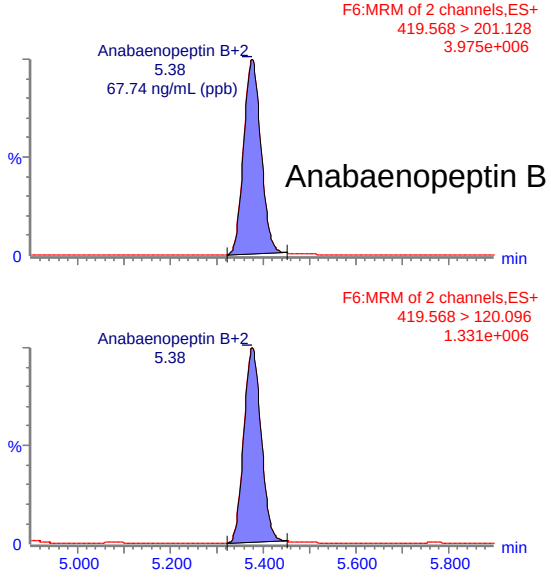
Example (Reservoir)

LC/MS/MS Results (ug/L)						
LR	RR	LW	LA	LF	DAsp-LR	Anabaenopeptin B
873.00	8.40	3.00	1073.00	14.00	29.30	67.70





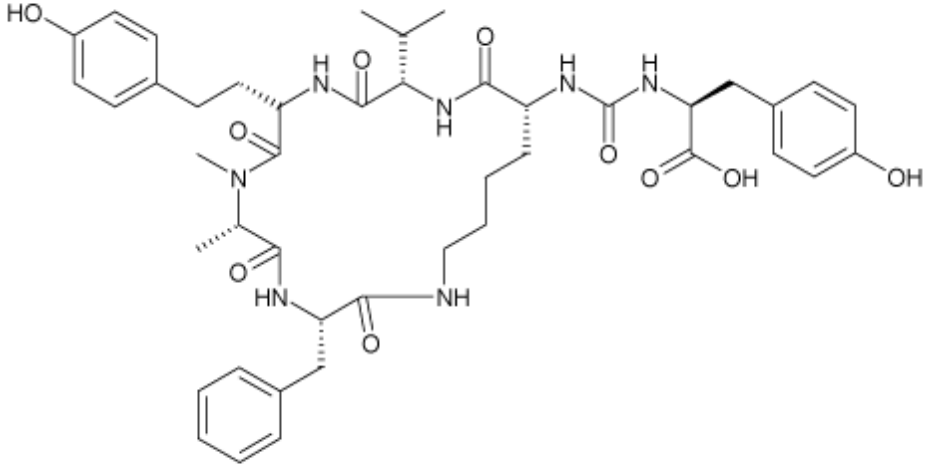




Anabaenopeptins

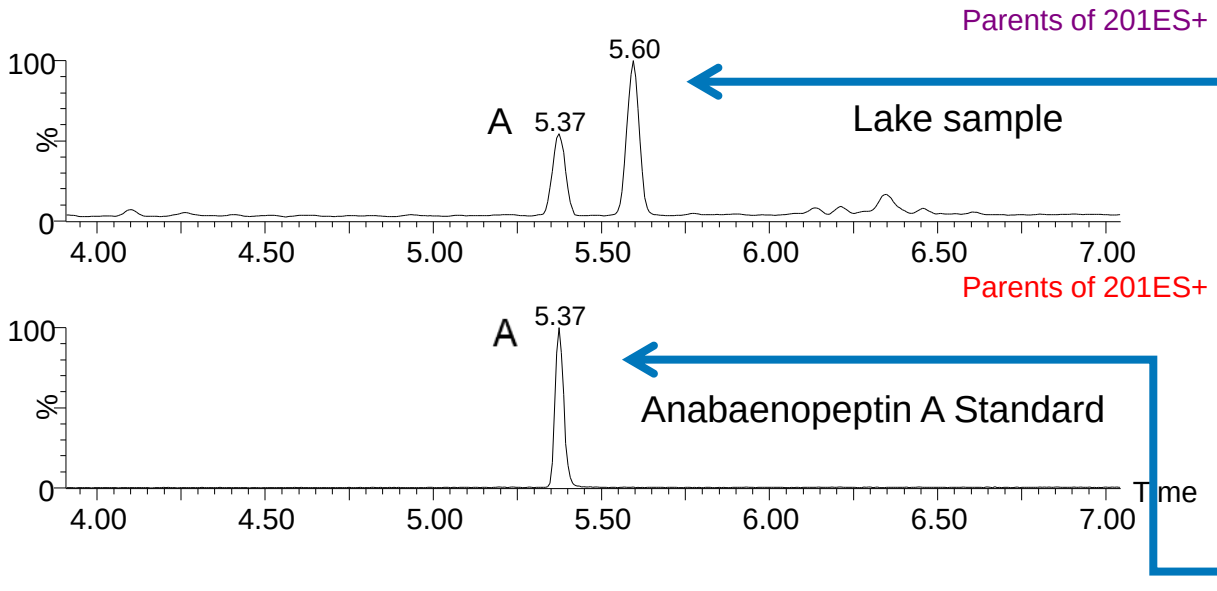
- Anabaenopeptins are a highly diverse group of bioactive peptides
- Produced by several genera of cyanobacteria such as *Anabaena, Planktothrix, Microcystis and Nodularia.*
- These peptides are commonly detected in cyanobacterial blooms along with the well known microcystins.

- Anabaenopeptin A

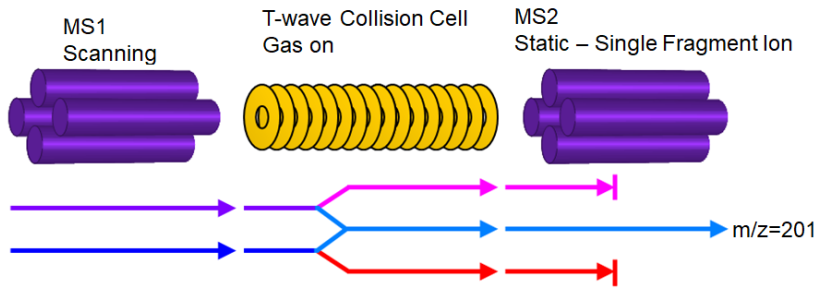


Finding more

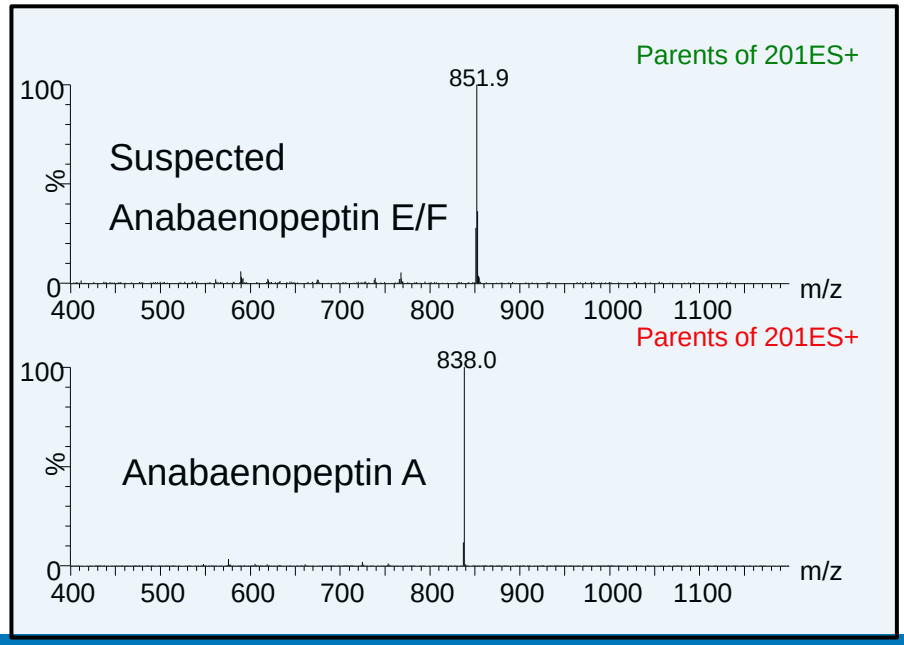
- Looking for other anabaenopeptins
  - A and B are available as standards.
  - Common fragment ion is m/z 201
    - $(CO\text{-}Arg)^{+1}$
  - Can scan for precursor (parent) mass



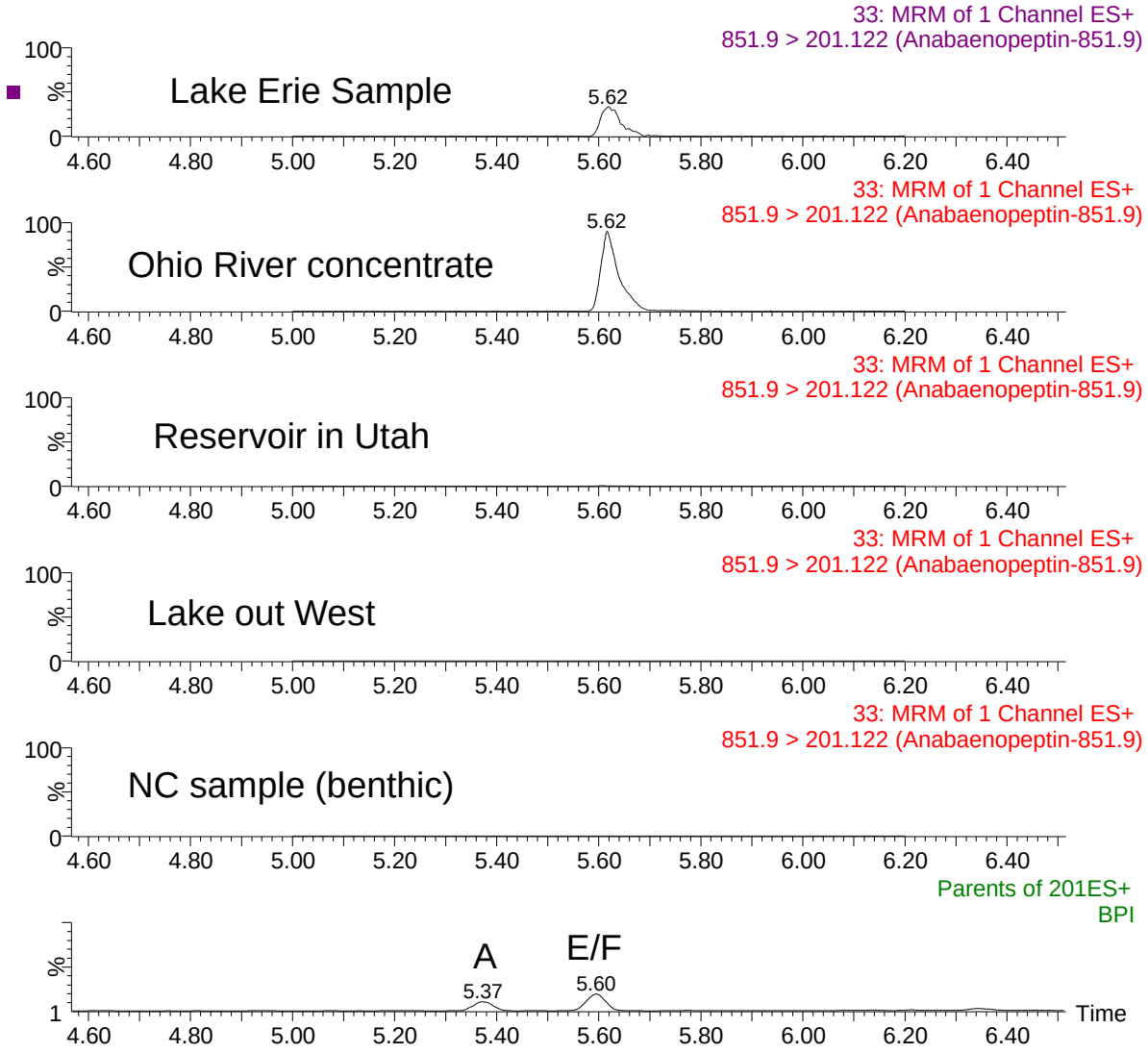
Precursor (Parent) Ion Scanning



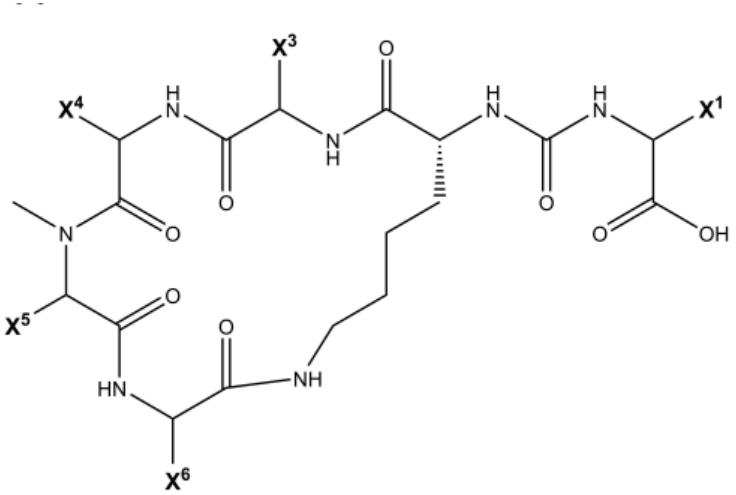
- MS1 is scanned over a specified mass range and all ions are sequentially passed through to the collision cell where they are fragmented
- MS2 is set to transmit only the mass of a specific fragment ion (i.e. m/z=201) and does not scan
- Any ions that fragment to give the specified product ion will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



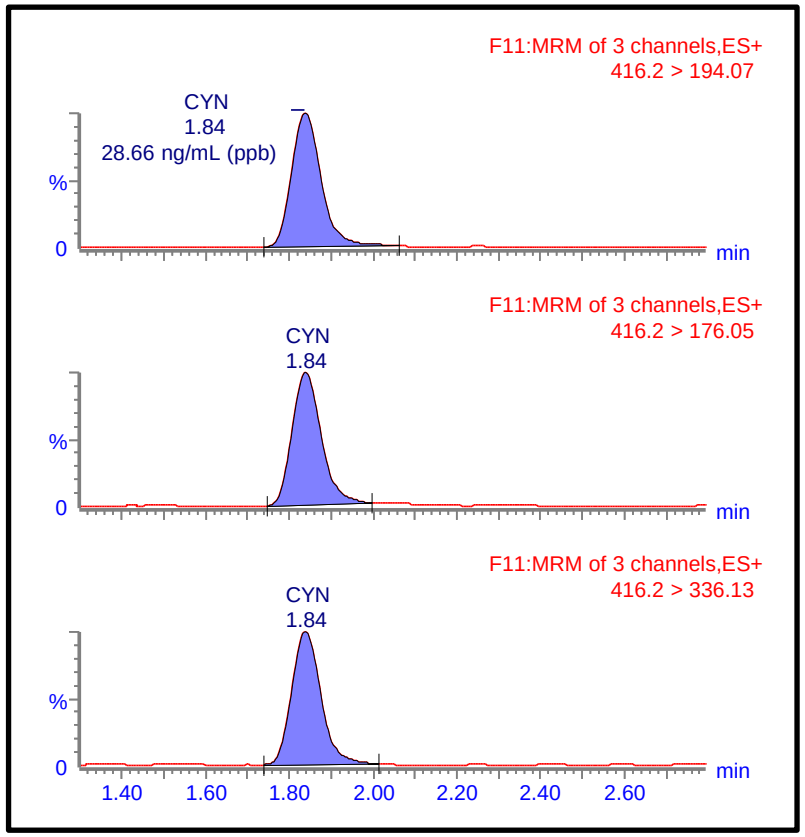
Monitoring for new compound

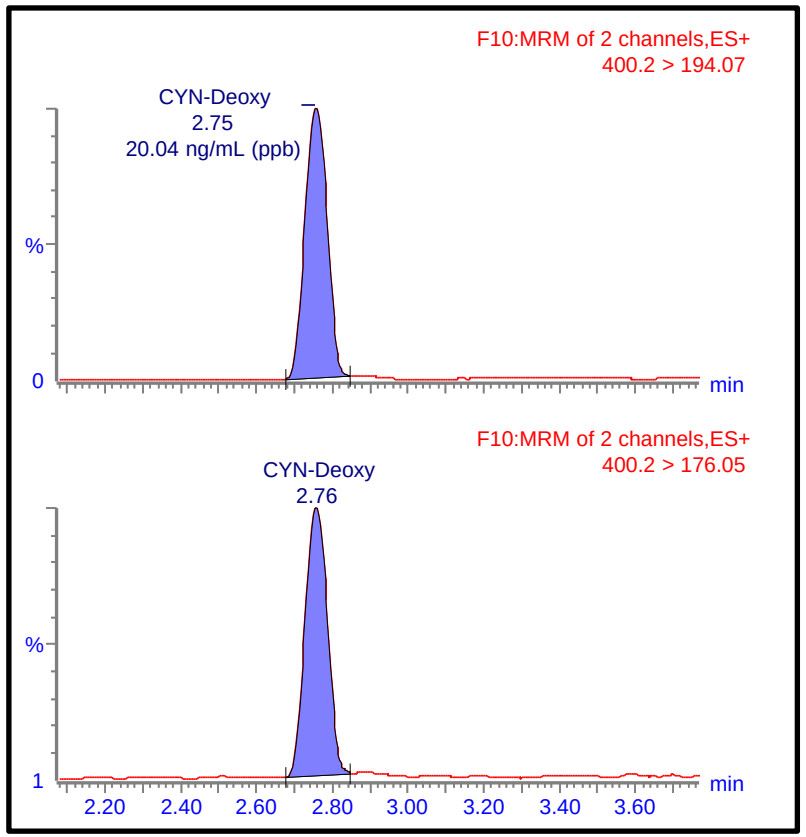


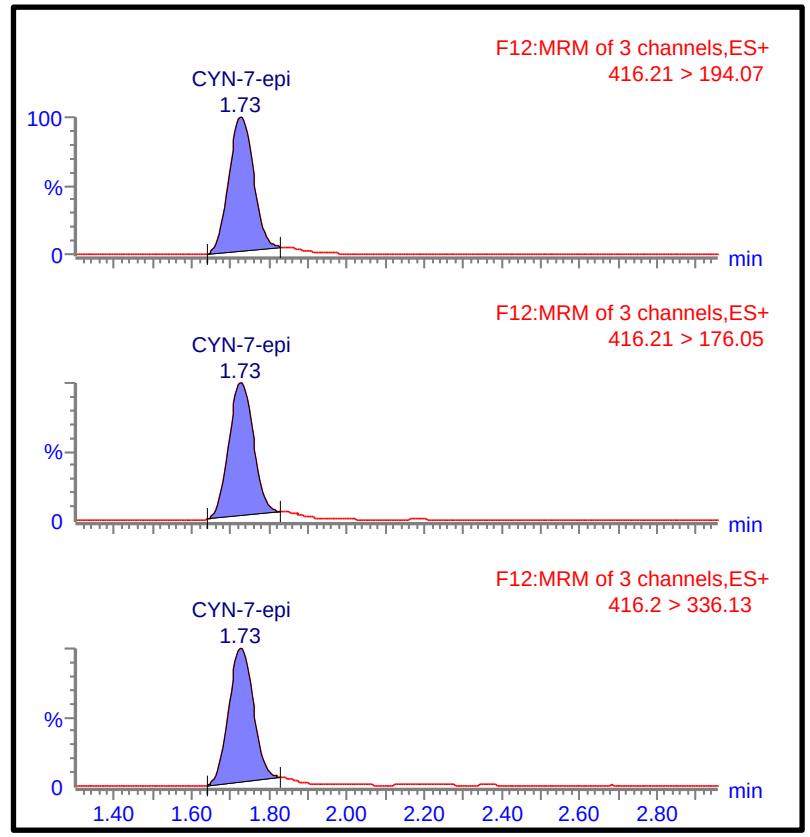
- Monitor for anabaenopeptin E/F



Additional Compounds (CYN analogs)

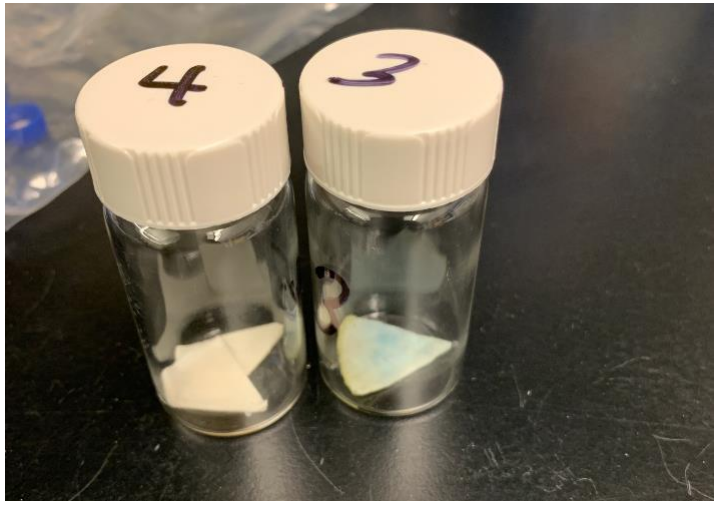




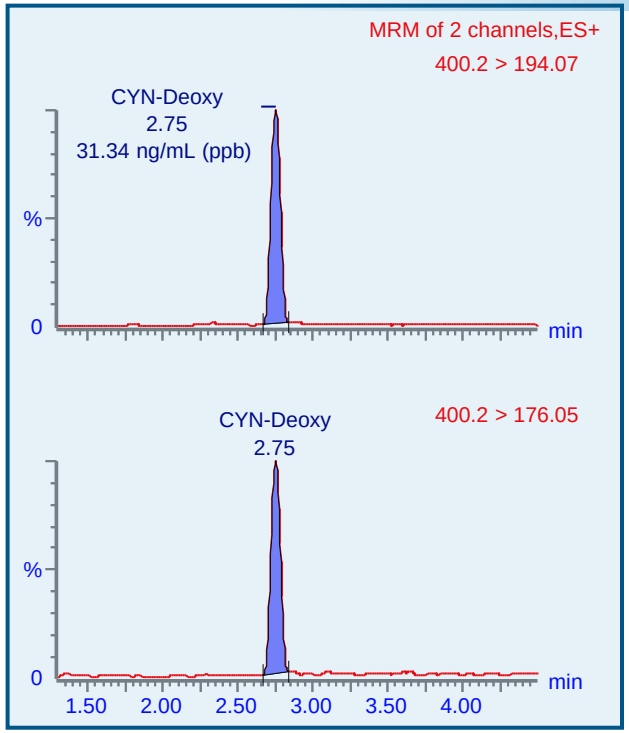


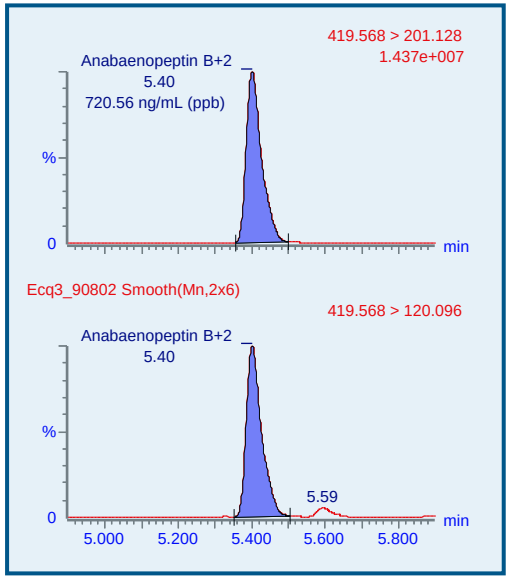
Ecuadorian Lake Sample

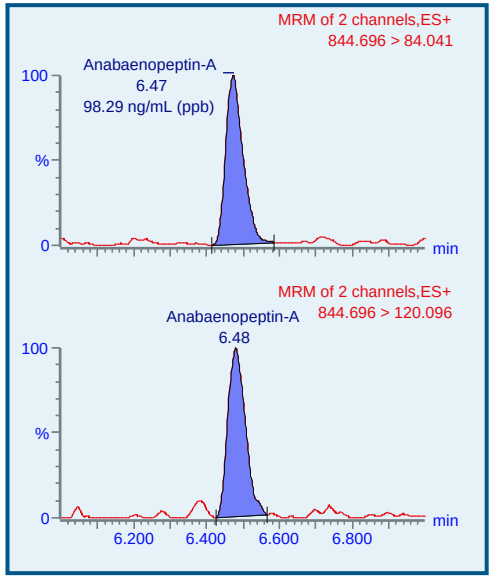
- Lake Yahuarcocha
- Filters were sent and extracted
- H2O/MeOH Extractions, filtered and analyzed

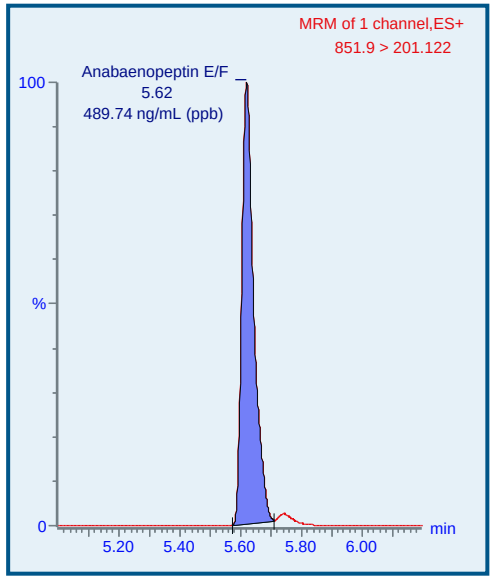


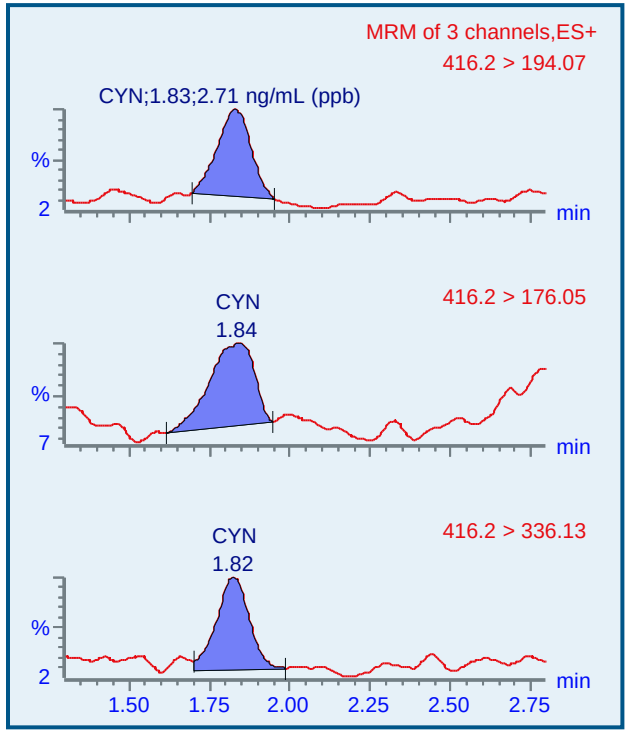
Ecuadorian Lake Sample











Sequencing Analysis

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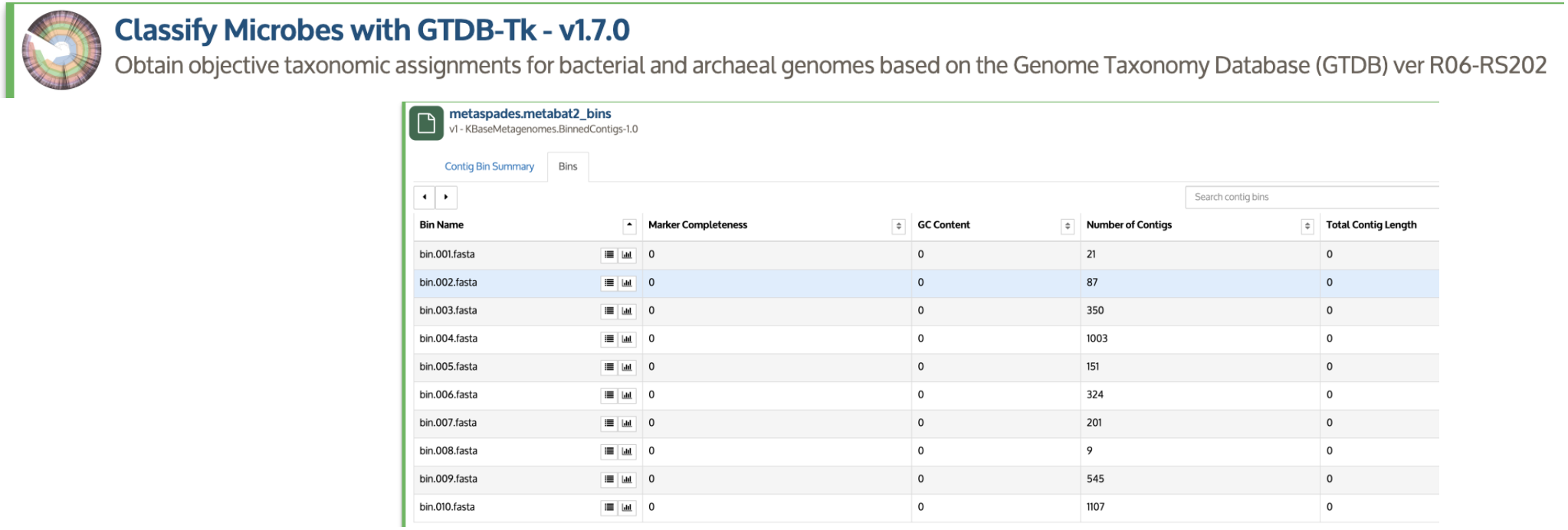
- We are sequencing the metagenome of filtered lake water.
  - If you sequence the whole DNA compliment of 1 organism it's the genome
    - if you sequence >1 organisms' genome in a sample you are sampling the metagenome.
- We are using KBase, which is run by Dept. of Energy as a web platform to use their large informatic computers to crunch this type of large data.
- A metagenome assembler is used that takes all the millions of randomly sequenced fragments from all the genomes of all the critters in the sample, and it uses statistical analysis and math to put it all back together.
- Then we use another statistical technique called metagenome binning, which is equivalent to thinking about dropping 50 boxes of different 1000 pieces puzzles, down the stairs and them all ending up in a large pile.
- The computer sorts through all the pieces looking for patterns/colors/matching fragments and tries to separate the puzzles back into their respective boxes.

Sequencing Analysis

- Then we determine the genome completeness of the bin.
- That tells us, if the bin contains the minimum number of genes known from all bacteria in that phylogenetic group (i.e. Cyanobacteria have 273 genes common to every sequenced cyanobacteria).
- If we have at least 272 of those cyanobacteria genes, then our completeness is 99.6%, and if there are more than 1 copy of genes that should only be there 1 time, we call that contamination (meaning it could be mis-binned, or could be an artifact of assembly



Bin Name	Marker Completeness	GC Content	Number of Contigs	Total Contig Length
bin.001.fasta	0	0	21	0
bin.002.fasta	0	0	87	0
bin.003.fasta	0	0	350	0
bin.004.fasta	0	0	1003	0
bin.005.fasta	0	0	151	0
bin.006.fasta	0	0	324	0
bin.007.fasta	0	0	201	0
bin.008.fasta	0	0	9	0
bin.009.fasta	0	0	545	0
bin.010.fasta	0	0	1107	0

Sequencing Analysis--Results

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Assess Genome Quality with CheckM - v1.0.18

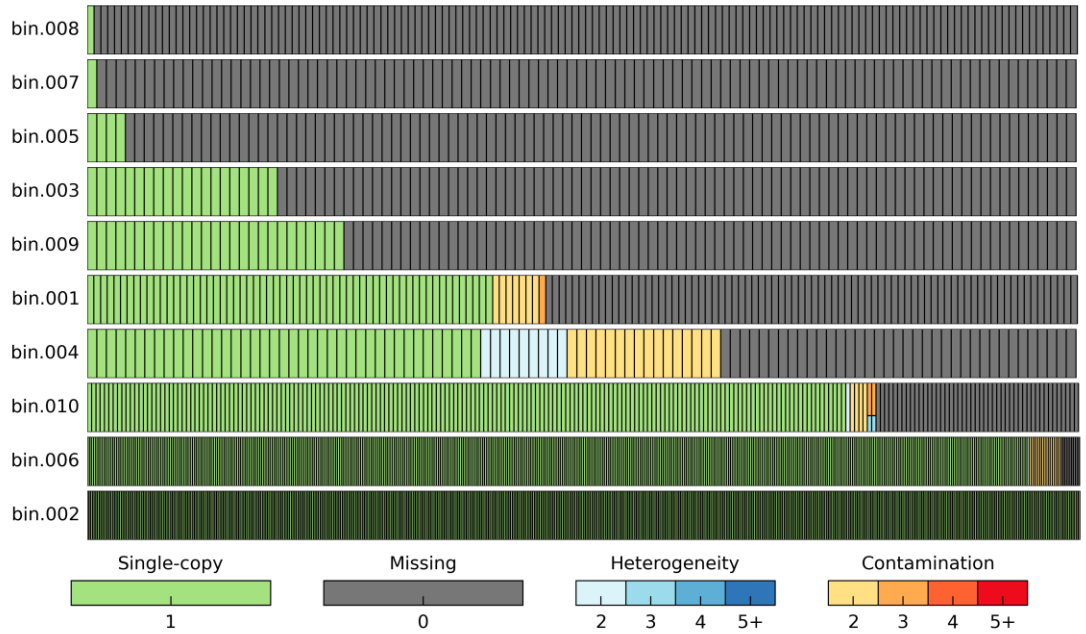
Runs the CheckM lineage workflow to assess the genome quality of isolates, single cells, or genome bins from metagenome assemblies through comparison to an existing database of genomes.

Bin Name	Marker Lineage	# Genomes	# Markers	# Marker Sets	0	1	2	3	4	5+	Completeness	Contamination
bin.001	k__Archaea	207	149	107	80	61	7	1	0	0	40.98	3.56
bin.002	p__Cyanobacteria	79	584	458	0	583	1	0	0	0	100.0	0.22
bin.003	k__Bacteria	5449	104	58	84	20	0	0	0	0	21.3	0.0
bin.004	k__Bacteria	5449	103	57	37	41	25	0	0	0	64.59	20.13
bin.005	k__Bacteria	5449	104	58	100	4	0	0	0	0	6.9	0.0
bin.006	o__Lactobacillales	294	472	265	9	447	16	0	0	0	97.95	3.94
bin.007	k__Bacteria	5449	103	58	102	1	0	0	0	0	1.72	0.0
bin.008	k__Archaea	207	145	103	144	1	0	0	0	0	0.97	0.0
bin.009	k__Bacteria	5449	104	58	77	27	0	0	0	0	31.65	0.0
bin.010	k__Bacteria	88	230	148	47	176	5	2	0	0	81.51	6.08

Bacteria | Archaea | Bacteria Marker Summary | Archaea Marker Summary

CSV | Column visibility

User Genome	Classification	FastANI Reference	FastANI Reference Radius	FastANI Taxonomy	FastANI ANI
bin.002.fa	d__Bacteria; p__Cyanobacteria; c__Cyanobacteriia; o__Cyanobacteriales; f__Microcoleaceae; g__Planktothrix; s__Planktothrix agardhii	GCA_003609755.1	96.3913	d__Bacteria; p__Cyanobacteria; c__Cyanobacteriia; o__Cyanobacteriales; f__Microcoleaceae; g__Planktothrix; s__Planktothrix agardhii	99.16

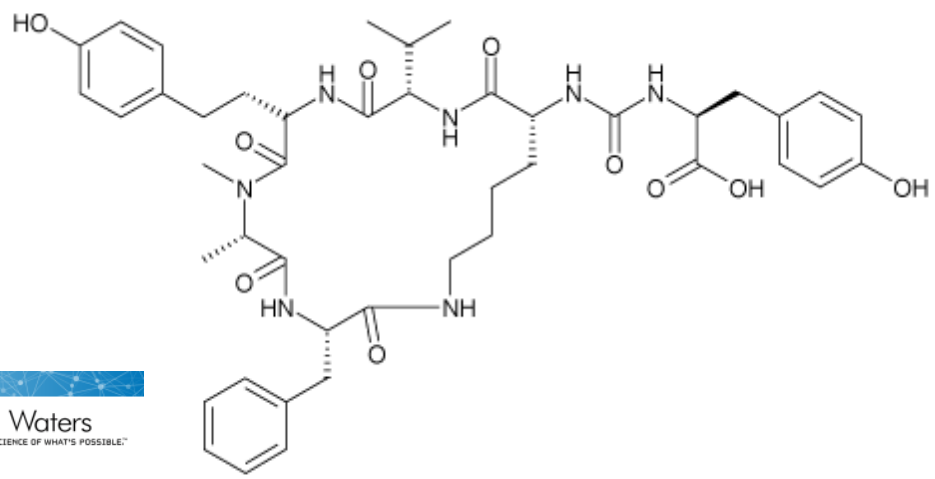


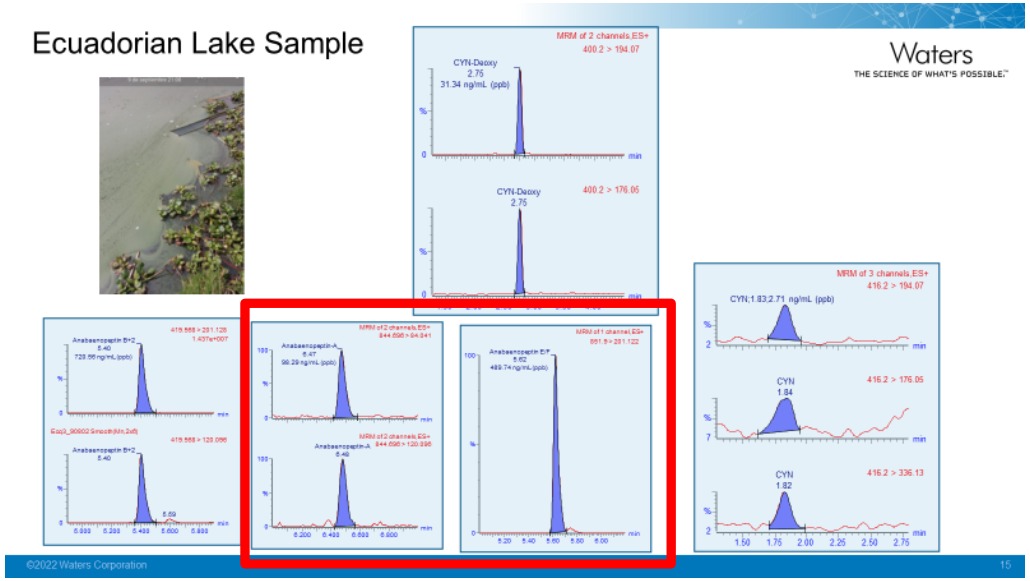
Now that we have the genome binned it represents in our case something at least 96% similar to *Planktothrix agardhii*, and then we can interrogate the genome by looking for toxin related proteins, domains, features.

Remember from Earlier
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





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Additional Work on Other Samples

Ohio River Bloom (September-October 2019)

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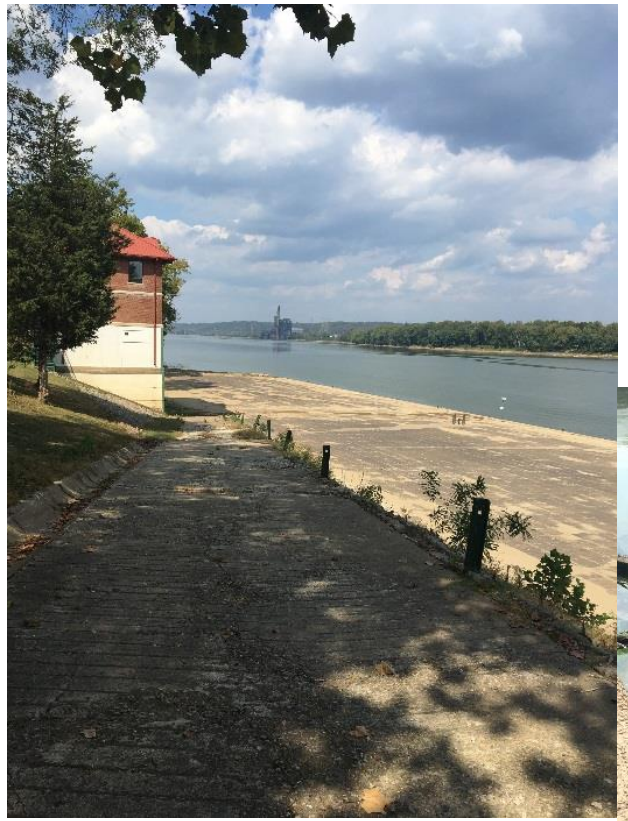
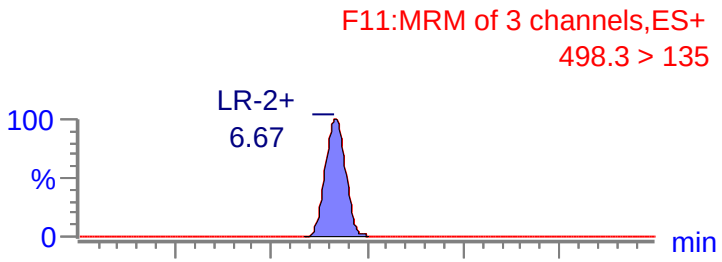
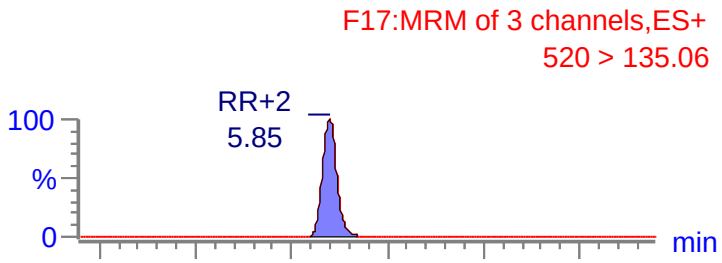
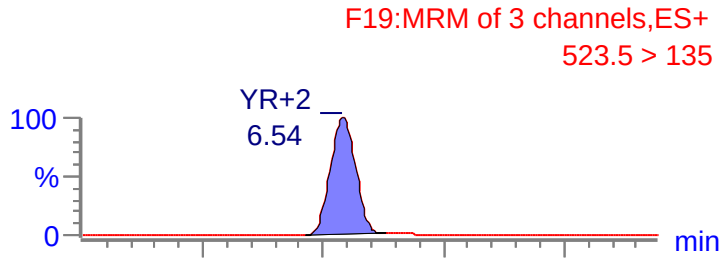
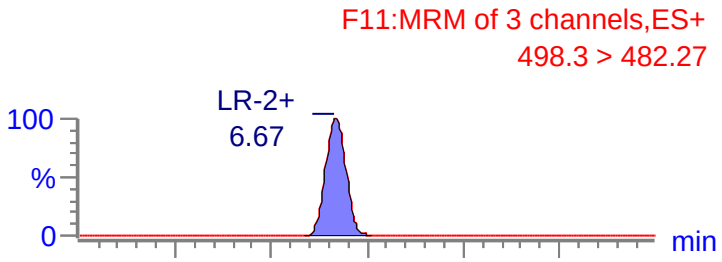


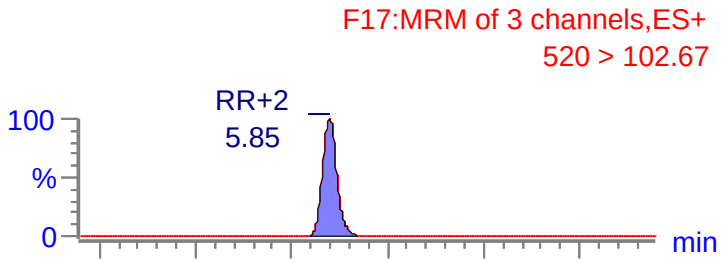
Results (freeze/thaw)

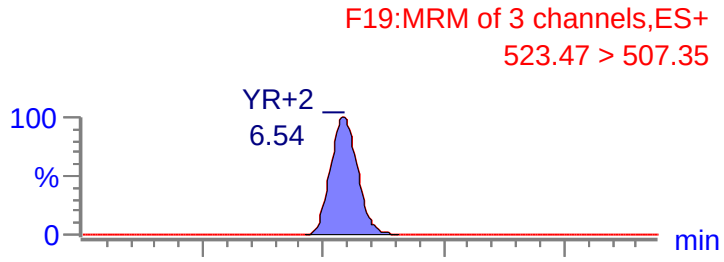


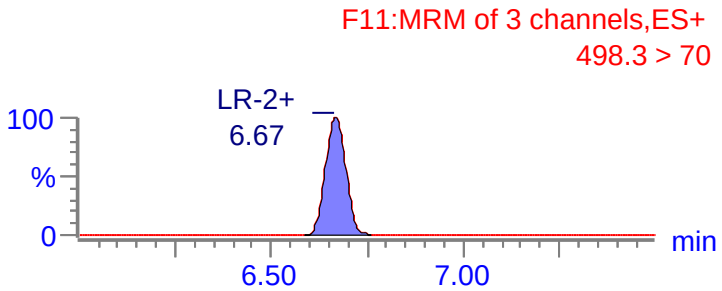


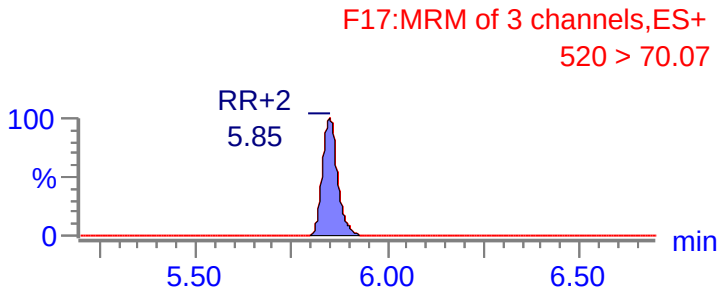


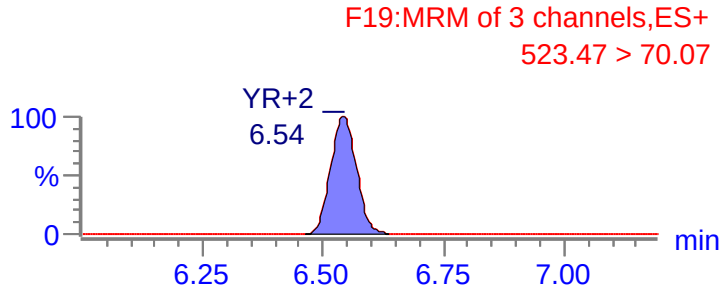












Also DAsp-LR, DAsp-RR, and Anabaenopeptin B

Interesting Screen (Precursor Scan (135 (ADDA))

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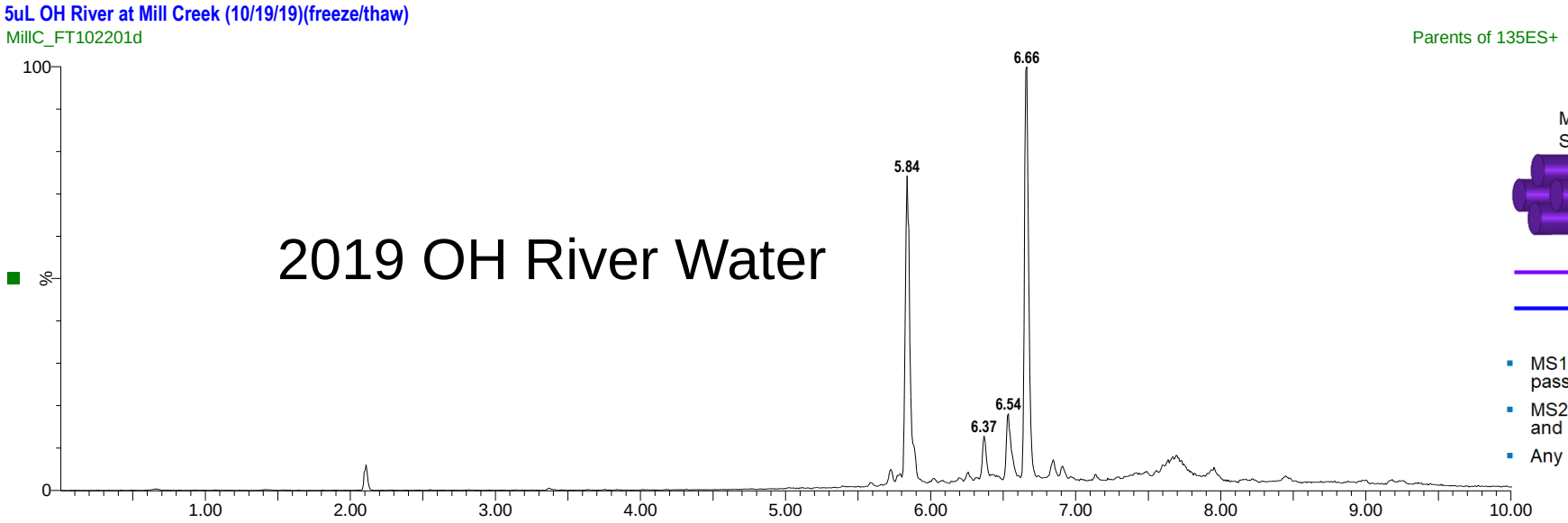

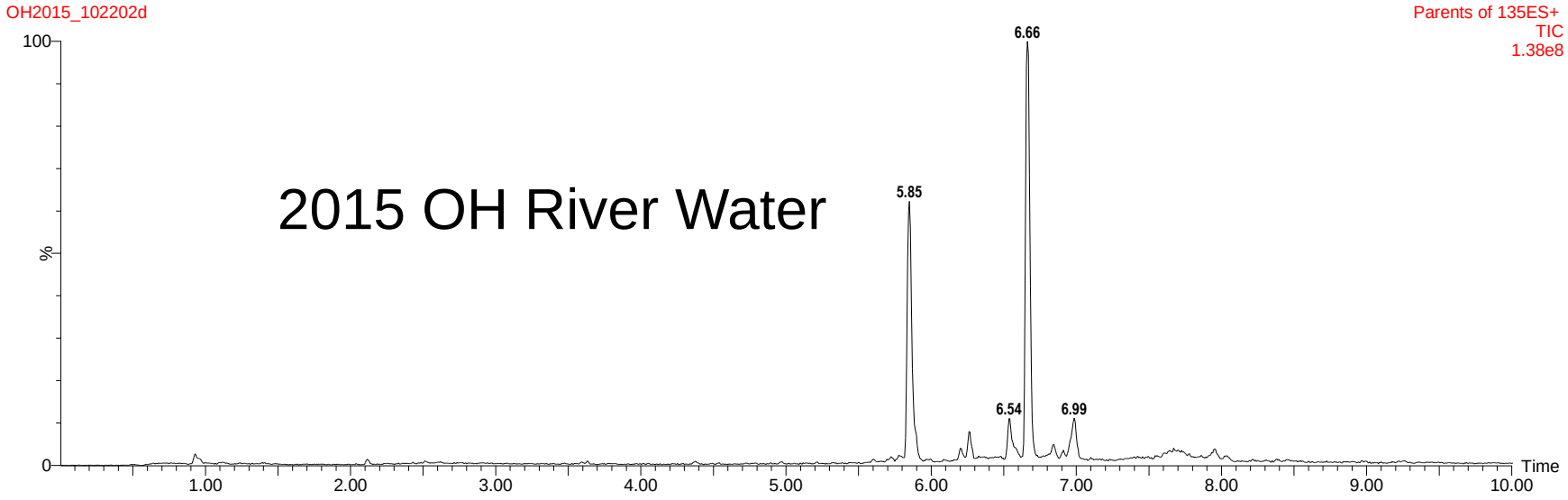

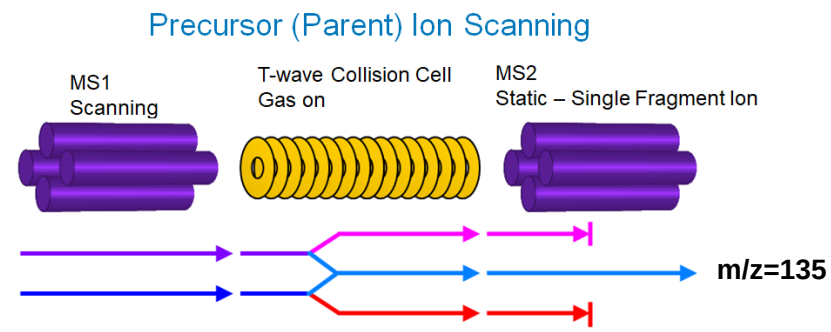

- MS1 is scanned over a specified mass range and all ions are sequentially passed through to the collision cell where they are fragmented
- MS2 is set to transmit only the mass of a specific fragment ion (i.e. m/z=201) and does not scan
- Any ions that fragment to give the specified product ion will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

Ohio Lake (2021)

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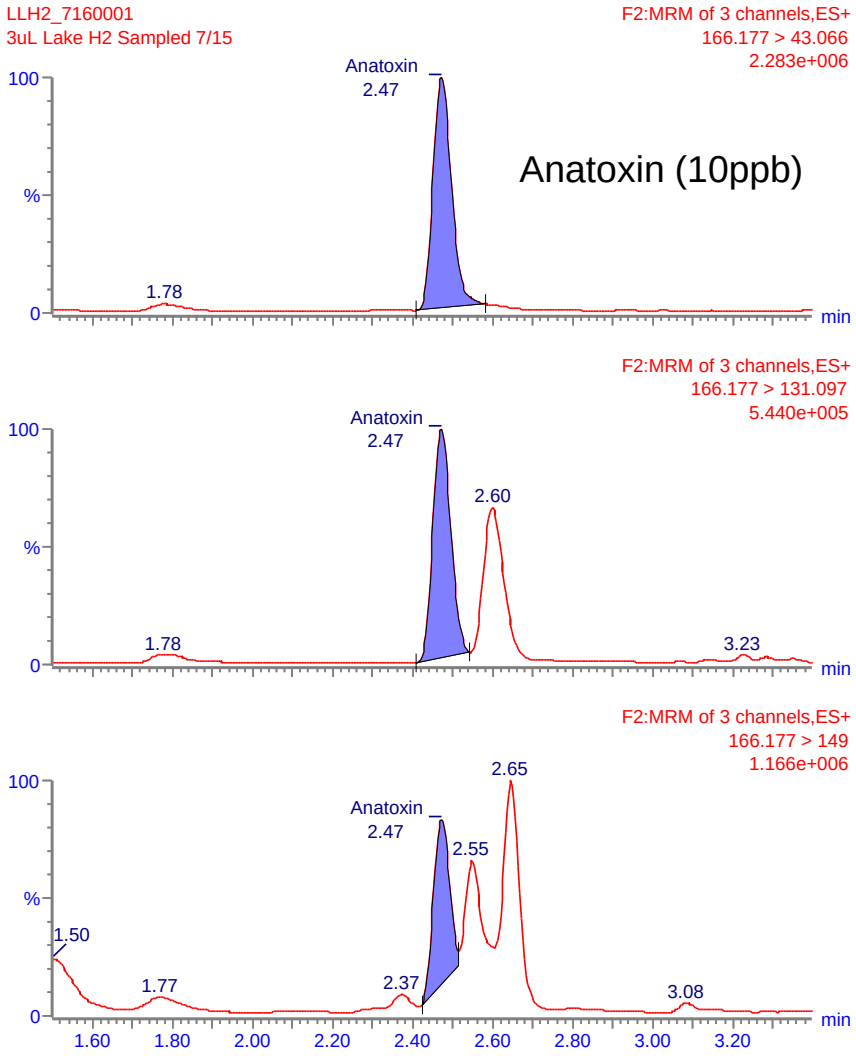

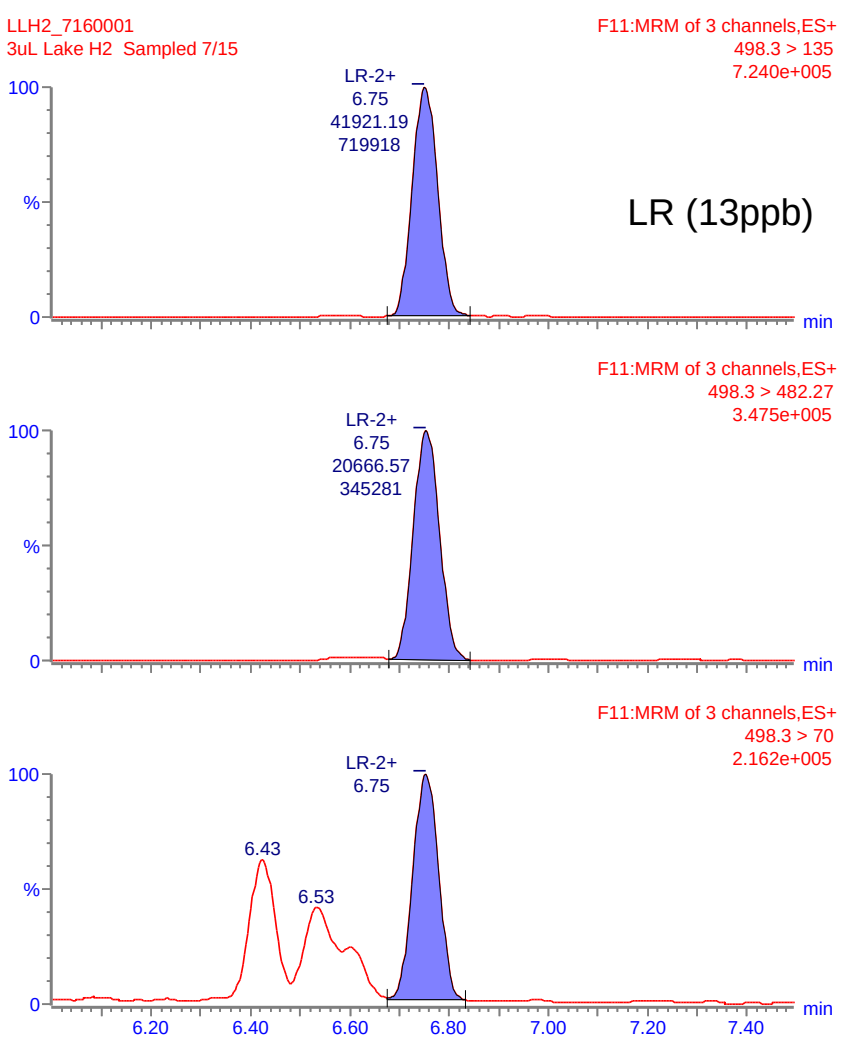

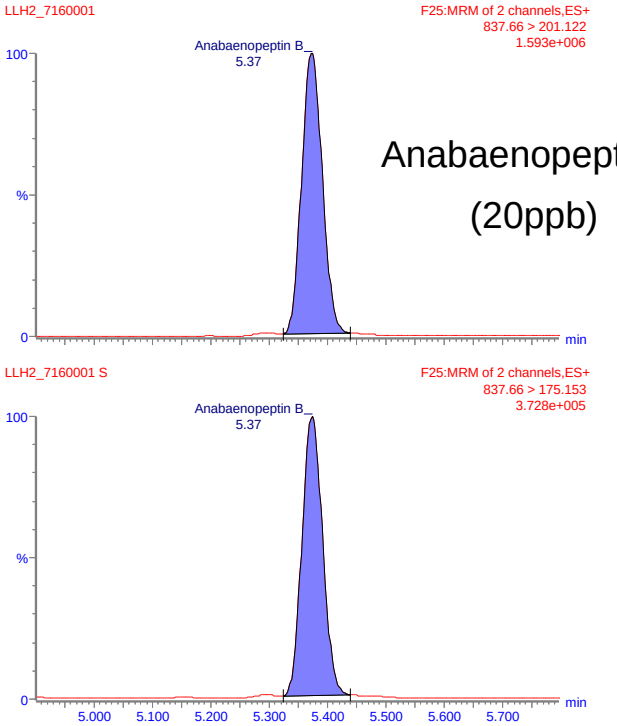

Summary

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- 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 and Persistent organic pollutants (POP's) for example).
- Questions? Interesting samples? Stuart_Oehrle@waters.com

Acknowledgements

- Waters Corporation
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- NKU Biology Department
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  - Maria de Lourdes Guerra

Thank You for Attending!!!