Expanding EPA 544:

Addition of Seven Microcystin Congeners for Analysis of Lake Erie Beach Samples, a Comparative Study with ELISA

Mark Citriglia, Manager of Analytical Services

Cheryl Soltis-Muth, Supervising Chemist
Northeast Ohio Regional Sewer District

Debbie Schordock, Advanced Instrumentation Chemist
Northeast Ohio Regional Sewer District

Northeast Ohio Regional Sewer District

Objectives

- Discuss the impact of HABs on Water & Wastewater utilities on Lake Erie
  - *Why we did what we did…*
- Discuss EPA Method 544 development and validation by NEORSD
  - *The good, the bad, and the ugly…*
- Discuss the comparative data between ELISA and LC/MS/MS results
- Discuss the method "expansion"
  - *Did it help?*
- Discuss next steps in our research

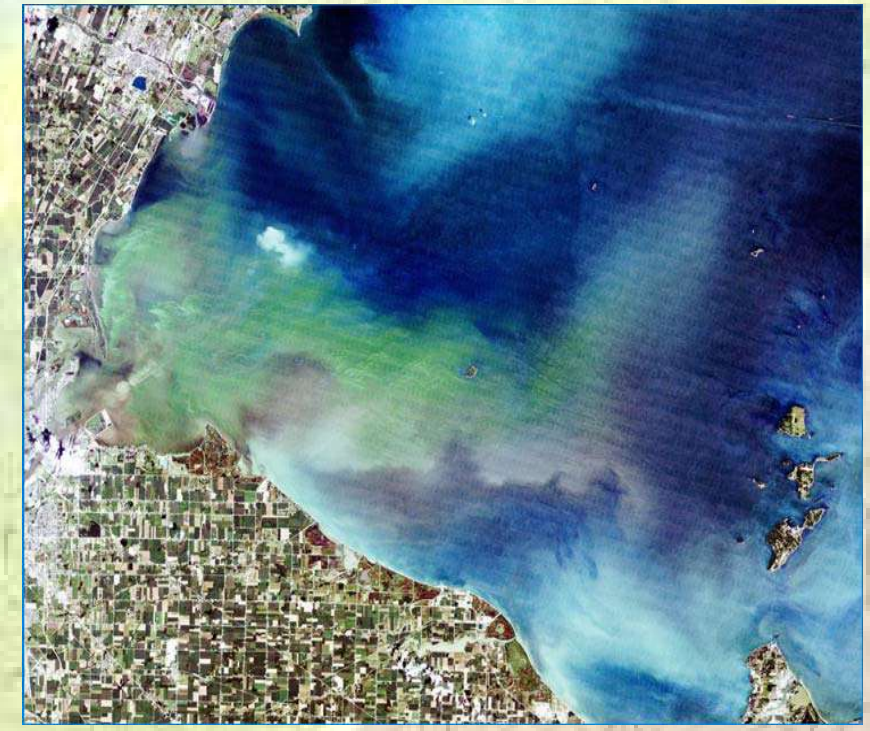
HAB History

Years	Event
1920-1964	Lake Erie algae biomass increases six-fold Diatoms replaced by cyanobacteria
1970-1990	Harmful algal blooms prompted creation of the GLWQA between the U.S. and Canada (1972) Phosphorus controls enacted Phosphorus controls lead to reduced algae biomass
Mid-Late 1990s	Return to eutrophic conditions Algae biomass begins to increase
2003	Return of harmful algal blooms – dissolved phosphorus conditions increasing
2011	Largest HAB to date
2014	City of Toledo, Ohio issues a "DO NOT DRINK" advisory on August 2 that lasted until August 4
2015	HAB that topped 2011 biomass

HAB History

- 2002-2003 – and so it begins…

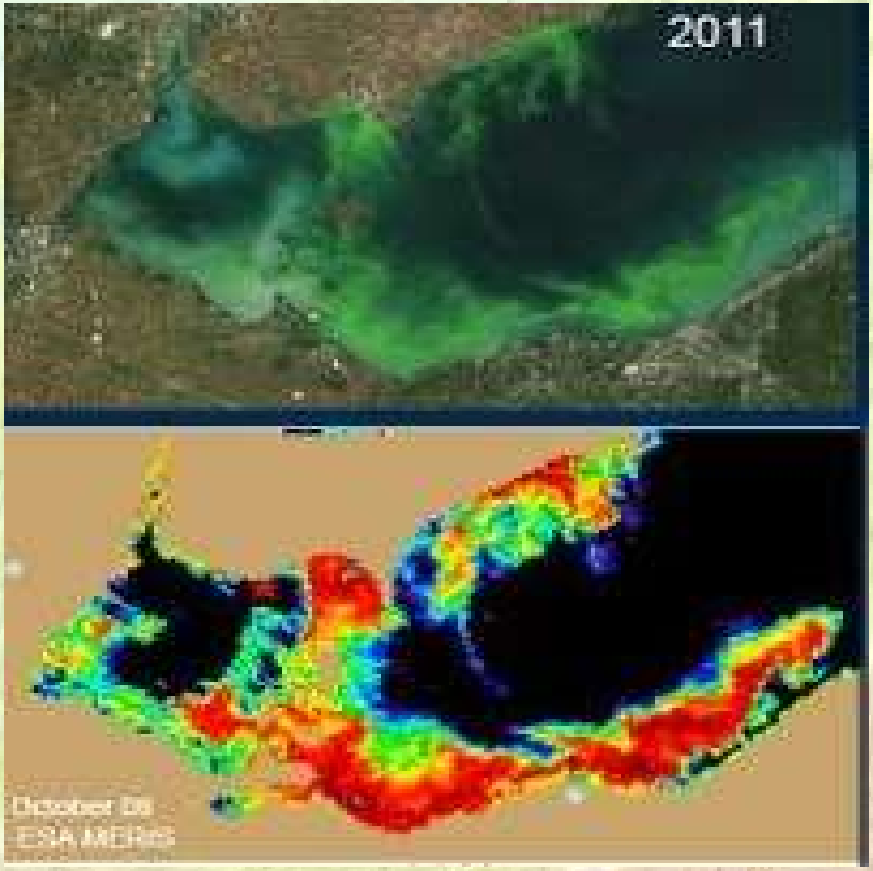
August 2002



August 2003

HAB History

- 2011 – A "Banner" Year



HAB History

- 2014 – A Water Crisis

THE BLADE

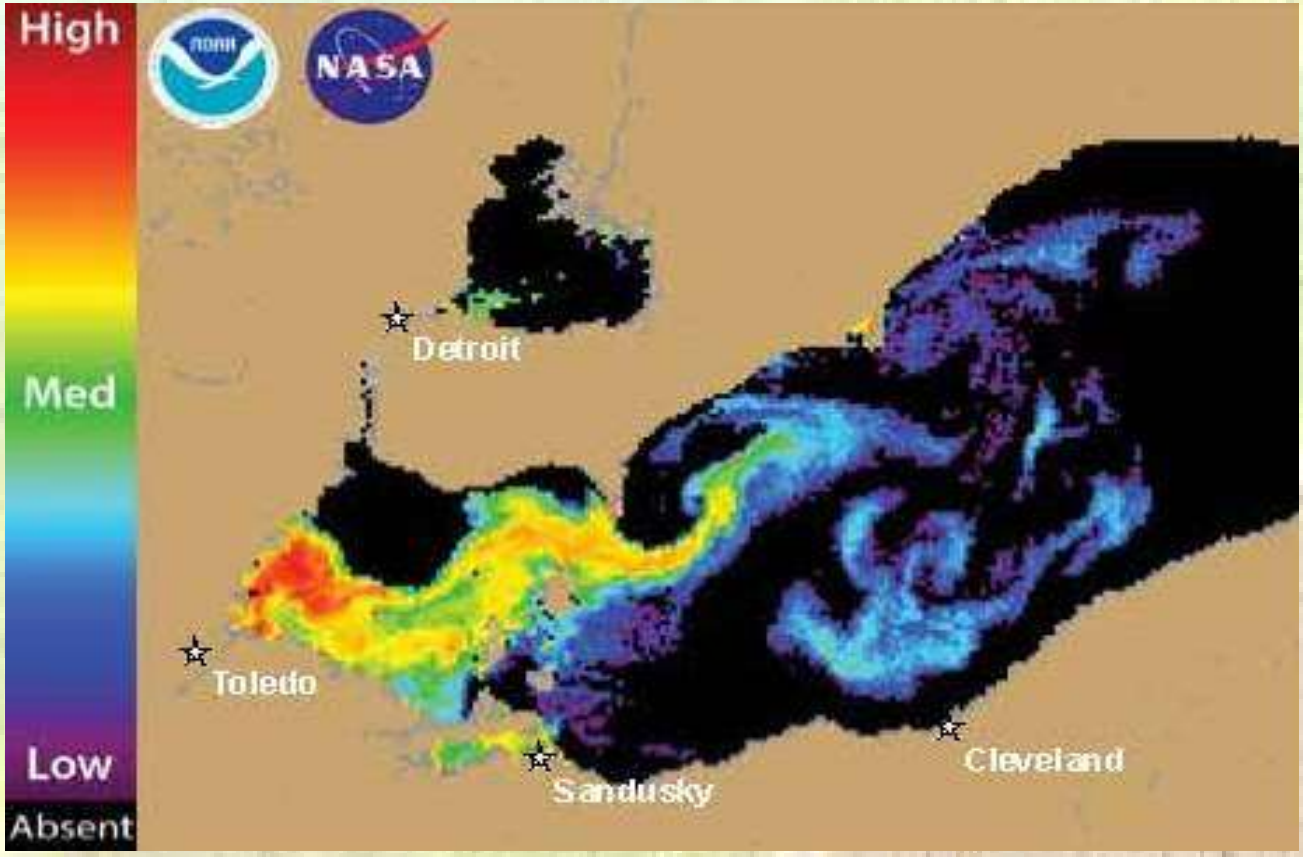
One of America's Great Newspapers

Toledo's water crisis

An algal toxin in Lake Erie contaminated the drinking water used by Toledo and many of its suburbs in August, 2014. It prompted a "do not drink" advisory for parts of three days and fueled public discussions about what created the problem and how to prevent it from happening again.

HAB History

- 2015 – A New Record – Covered over 300 square miles



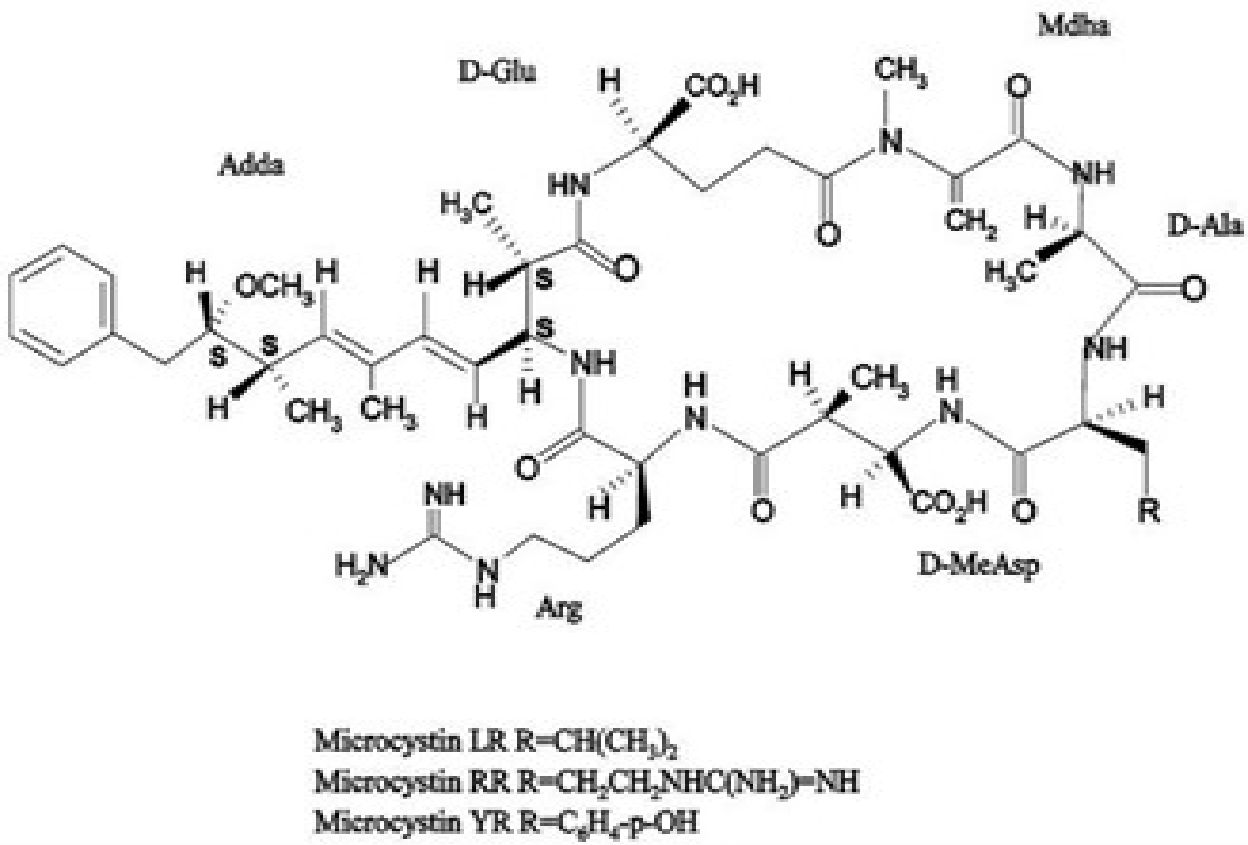
Expanding EPA 544

EPA Method 544

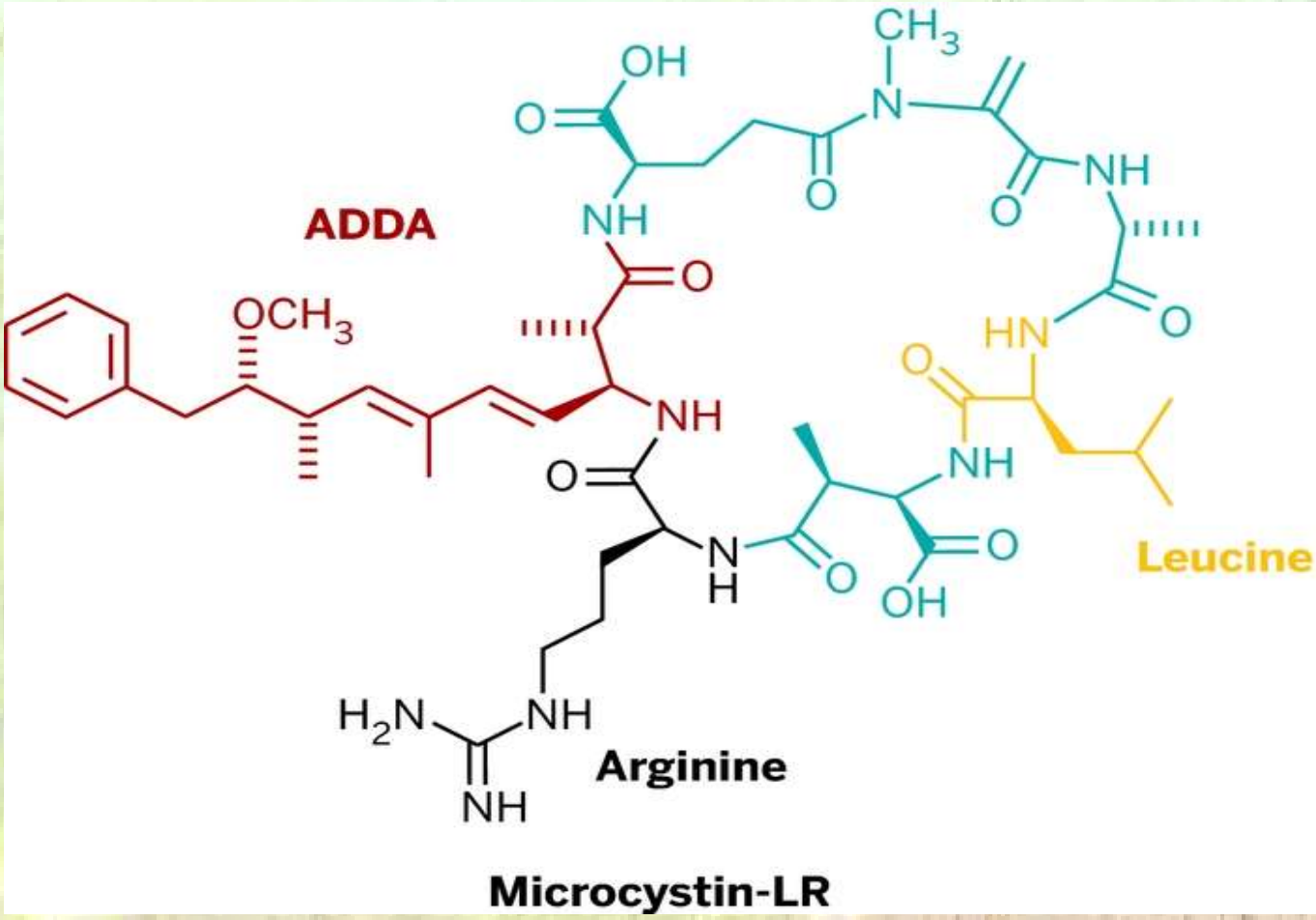
- February 2015, *EPA Method 544: Determination of Microcystins and Nodularin in Drinking Water by Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS)* was promulgated.

Analyte Name	Quant MRM	Calibration Range	Retention Time
Nodularin	825.20>135.10	0.25-100 ppb	11.764
Microcystin-YR	523.30>135.05	0.25-100 ppb	11.765
Microcystin-RR	519.80>135.00	0.25-100 ppb	11.989
Microcystin-LR	498.40>135.15	0.25-100 ppb	12.255
Microcystin-LA	910.30>776.45	0.25-100 ppb	13.209
Microcystin-LY	1002.30>135.10	0.50-100 ppb	13.244
Microcystin-LF	986.40>135.10	0.50-100 ppb	14.805
Surrogate	514.90>135.25	NA	14.999

EPA Method 544



EPA Method 544



EPA Method 544

- NEORSD was positioned to validate the method quickly in part because of help provided by Dr. Judy Westrick, Director of the Lumigen Instrument Center at Wayne State University in Detroit, Michigan and her staff, most notably, Dr. Johnna Birbeck.
  - Solid phase extraction (SPE), sample concentration, and injection into LC/MS/MS for separation and quantitation
  - Intracellular and extracellular toxins
  - The method allows for some flexibility
    - Reduced sample collection volume from 500-mL to 100-mL.

Validation *The Good*

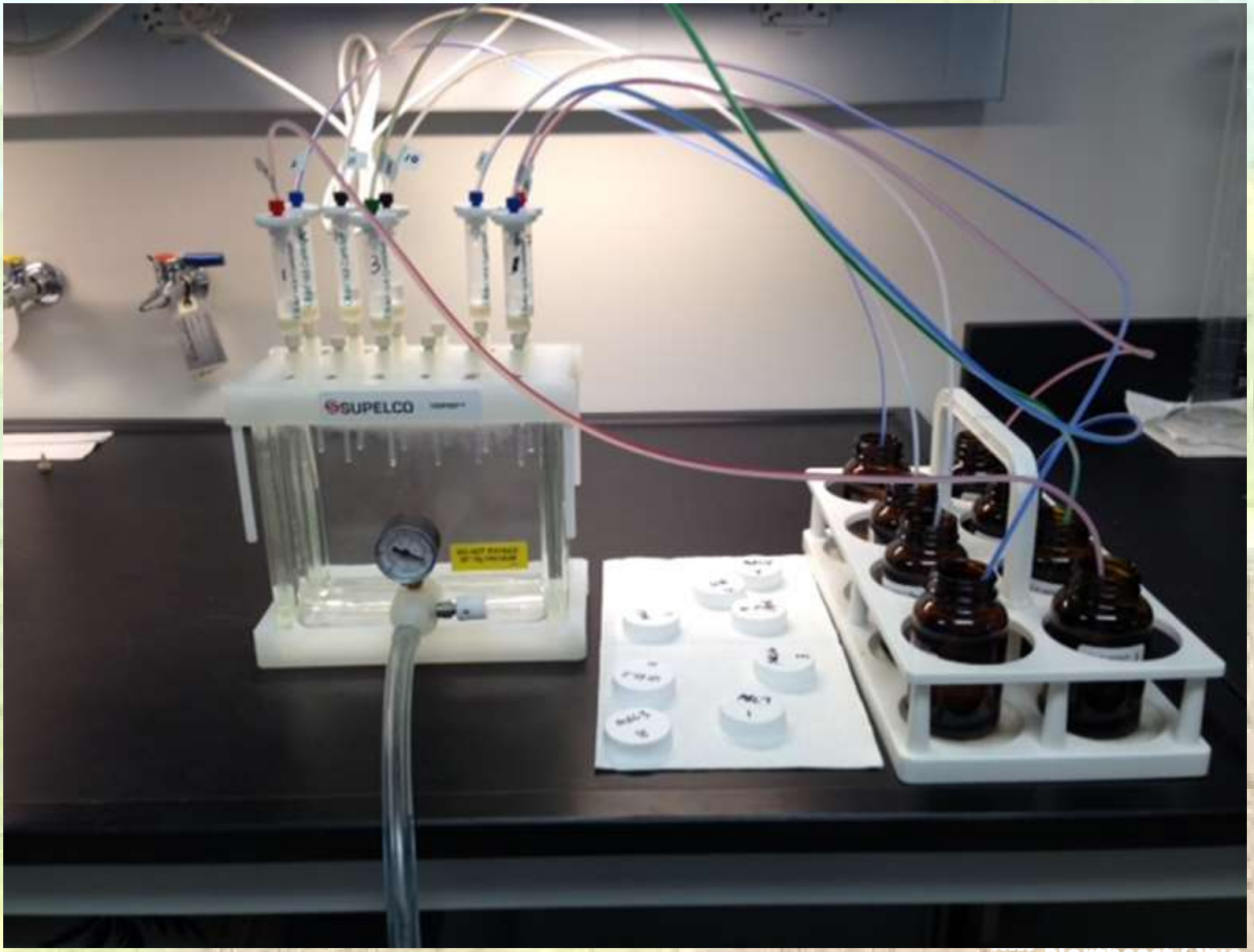
- 100-mL samples
  - Amber glass jars - Teflon-lined lids
  - Preservation
    - Trizma (a buffering reagent)
    - 2-Chloroacetamide (an antimicrobial
    - Ascorbic Acid (a dechlorinating agent)
    - EDTA (to inhibit binding of targets to metals)

NOTE: The preservatives are for drinking water samples, but because validation of method performed using the preservatives and method also states that sample collection cannot be altered, they were kept in the method for environmental samples.

Validation *The Good*

- Entire 100-mL filtered using Nuclepore filters. Filtrates retained. Filters placed in freezer for 1-16 hours in 80% MeOH in water. Liquid from filters drawn off and added back to retained filtrates.
- Solid phase extraction was carried out on a Visiprep™ SPE Vacuum Manifold using Waters Oasis HLB, 150 mg, 6cc cartridges
- SPE extract was concentrated to dryness under nitrogen and reconstituted to 1 mL in 90% LCMS grade MeOH.

Expanding EPA 544



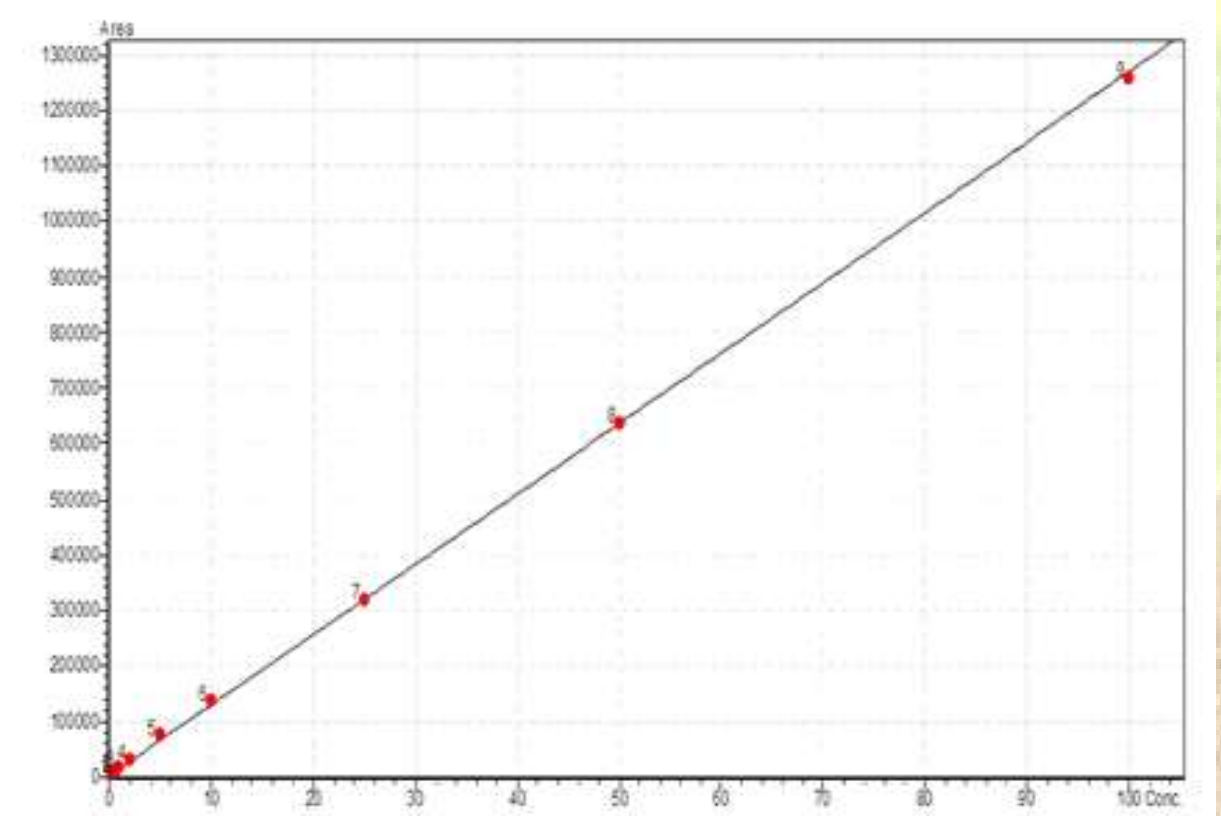
Northeast Ohio Regional Sewer District

Validation *The Good*

- Column: Phenomenex Kinetex C8 column, 2.6 µm, 2.1 x 100 mm
- Mobile Phase A: 20 mM Ammonium Formate
- Mobile Phase B: 100% LCMS Grade Methanol
- Flow Rate 0.3 mL/min
- Sample run time 26 min
- Injection volume 3-µL

Validation *The Good*

- Calibration curve for MC-LR, 9-point curve from 0.25-100 ppb.



Validation *The Good*

- All target analytes were verified with a second source standard.

- IDP
  - Extracted and analyzed four replicates of FBs which were preserved exactly as samples and spiked at 50 ppb with each analyte.
  - In each case, the relative standard deviation (RSD) was less than 30%.
- IDA
  - Used the results from the same set of FBs.
  - Calculated recoveries within ± 30% of the true value.

Validation *The Good*

- MRLs
  - Determined by fortifying, extracting, and analyzing seven replicate FBs at 20 ppt with same sample preservatives.
  - The mean concentration calculated as was the standard deviation.
  - The half range for the prediction interval of results (HR_{PIR}) was determined using the equation:

$$HR_{PIR} = 3.963s$$

s = standard deviation

3.963 = constant for 7 reps

Validation *The Good*

- MRLs *continued*
  - Once HR_{PIR} determined, confirmed that the upper and lower limits for the prediction interval of result (PIR = Mean + HR_{PIR}) met the upper and lower limits using the equations, respectively:

$$\frac{\text{Mean} + HR_{PIR}}{\text{Fortified Concentration}} \times 100\%$$

$$\frac{\text{Mean} - HR_{PIR}}{\text{Fortified Concentration}} \times 100\%$$

Validation *The Bad*

- While the method was validated quickly, NEORSD staff did encounter some problems.
- The main issue was **low standard and surrogate recoveries** in extracted samples.

Validation *The Bad*

- What is a surrogate?
  - Ethylated MC-LR, d_5 (C_2D_2-MC-LR)
  - Literally:
    - *A compound that has properties similar to the targets.*
    - *Not expected to be in environmental field samples and does not interfere with the identification or quantification of the targets.*

Validation *The Bad*

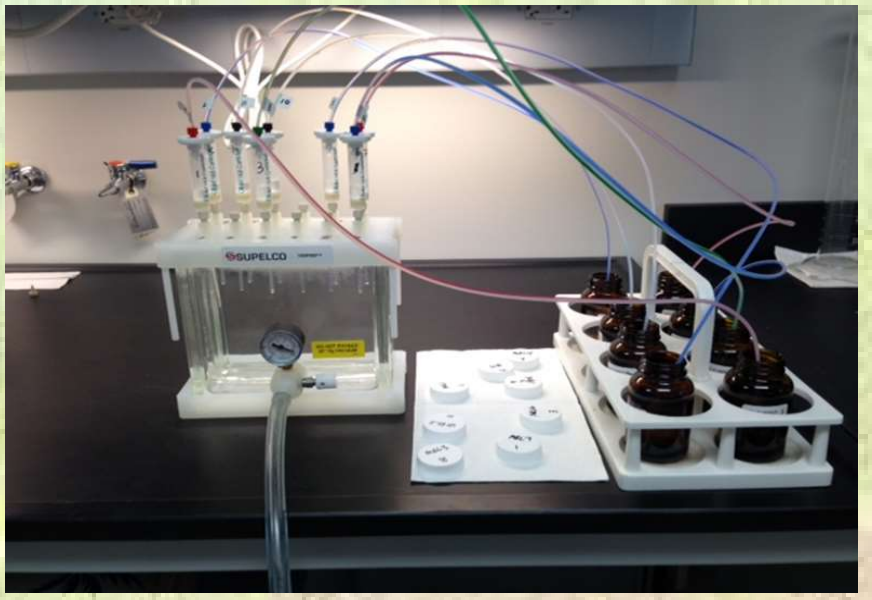
- *Surrogate recovery from the sample matrix serves a QC function on the suitability of the analytical method and the ability of the laboratory to perform the method with proficiency.*
- *If a surrogate compound is not recovered, an analyte of concern also may not be recovered.*

Validation *The Bad*

- Surrogate versus internal standard
  - *Internal standard provides a reference concentration against which the responses of the targets are compared.*
  - *Internal standard is added to the sample just prior to instrumental analysis so amount is consistent in all standards and samples and not dependent on extraction/concentration or other sample handling procedures.*
  - *The internal standard compensates for relatively minor fluctuations in instrument sensitivity to provide more accurate quantification of the targets.*

Validation *The Bad*

- Early extractions showed low surrogate recoveries.
  - Analyzed separate steps of the preparation process to determine where the surrogate was being lost.



Validation *The Bad*

- Evaporation Step – first step investigated
  - *Added10 mL of 90% MeOH to a test tube, spiked, evaporated, reconstituted with 1 mL MeOH, and analyzed.*
  - *Small loss at this step.*
  - *Improvement made by rinsing down the walls of the test tube part way through evaporation.*

Validation *The Bad*

- Extraction Step – next investigated
  - *Spiked without initial filtration was examined. Extraction/evaporation was very good, recoveries were all within 80% of true value. Thus, indications pointed to surrogate loss taking place in the intracellular toxin release filtration step.*

  - *It appeared that the surrogate was "sticking" to the glass of the filtration apparatus.*

  - *Adjusted the filtration process as follows:*

Validation *The Bad*

- *Rinsed bottle with 5 mL and 2.5 mL of ultrapure water.*
- *Poured the filtrate back into the rinsed sample bottle.*
- *Reassembled the funnel and rinsed the funnel and filter with the 5.0 mL and 2.5 mL of 90% MeOH.*
- *Removed the filter and swirled the 7.5 mL 90% MeOH in the flask to rinse all of the sides well and poured this into the sample bottle.*
- *This modification has greatly improved surrogate recoveries.*

Northeast Ohio Regional Sewer District

Validation *The Bad*

- In addition to these investigations, sample bottle and cartridge elution step from the method was changed slightly.

- *Prior to elution, the 90% MeOH is allowed to sit on the filter for 5 minutes before elution is continued.*
- *This was suggested by Dr. Jody Shoemaker to improve recoveries. Thanks!*

Validation *The Bad*

Sample Name	Surrogate recovery
Extracted blank	68.65%
Extracted standard 1	74.37%
Extracted standard 2	73.86%
Extracted standard 3	67.12%
Extracted standard 4	75.84%
Evaporation only	92.35%
SPE, evaporation only	92.22%

Validation *The Ugly*

- The second issue: Enhance recoveries in extracted standards versus un-extracted standards.
  - *Initially tried to develop a shorter sample run time. Developed a 14-minute run with good results using standards that had not been extracted. When extracted standards run, high recoveries in LA, LY, LW, LF.*
  - *When gradient extended to match the 26-minute run time of 544, the same extracts had more reasonable recoveries.*
  - *Future work includes spending more time on this issue to shorten the run time without the enhancement effects.*

Validation *The Ugly*

100 ppb not extracted – 14 minute run				
ID#	Name	Ret. Time	Conc.	Unit
1	RR	4.703	103.71	ppb
2	YR	5.258	100.58	ppb
5	LR	5.366	101.18	ppb
6	LA	6.665	102.90	ppb
7	LY	6.747	102.29	ppb
8	LW	7.272	101.43	ppb
9	LF	7.452	101.19	ppb

100 ppb extracted - 14 minute run				
ID#	Name	Ret. Time	Conc.	Unit
1	RR	4.702	97.76	ppb
2	YR	5.258	70.64	ppb
5	LR	5.365	79.64	ppb
6	LA	6.657	189.54	ppb
7	LY	6.735	138.70	ppb
8	LW	7.259	143.44	ppb
9	LF	7.435	145.94	ppb

Northeast Ohio Regional Sewer District

Validation *The Ugly*

100 ppb not extracted - 20 minute run

ID#	Name	Ret. Time	Conc.	Unit
1	RR	11.741	100.28	ppb
2	YR	11.543	99.282	ppb
5	LR	12.025	99.313	ppb
6	LA	12.972	99.747	ppb
7	LY	13.03	99.352	ppb
8	LW	13.975	100.058	ppb
9	LF	14.566	99.781	ppb

100 ppb extracted - 20 minute run

ID#	Name	Ret. Time	Conc.	Unit
1	RR	11.742	91.368	ppb
2	YR	11.542	93.984	ppb
5	LR	12.027	102.213	ppb
6	LA	12.973	111.437	ppb
7	LY	13.03	79.222	ppb
8	LW	13.968	95.934	ppb
9	LF	14.568	105.902	ppb

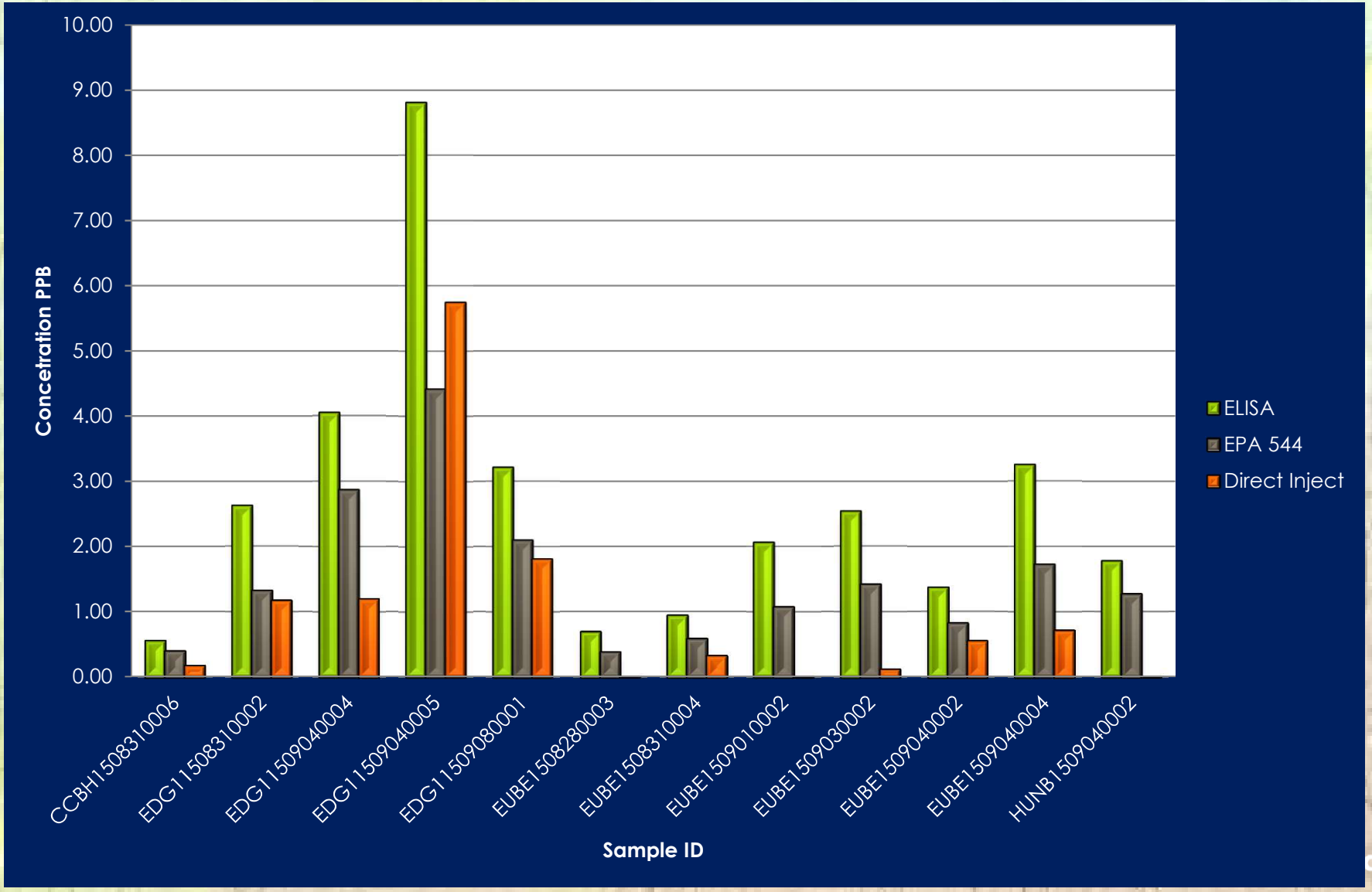
Looking at the Data

- Summer of 2015 Work
  - NEORSD collected samples to analyze using EPA 544 and ELISA for total microcystin.
  - EPA 544 targets six of the 100 or so microcystin congeners currently identified.
  - Analyzed **37** Lake Erie beach water samples
  - The samples analyzed for ELISA were lysed by a series of three freeze/thaw cycles and filtered.
  - The resulting lysed filtrates from ELISA were also analyzed by directly injecting into the LC/MS/MS with the same operating conditions as with EPA Method 544.

Looking at the Data

- In 18 cases the 544 data was greater than the ELISA data for total microcystins.
- In 19 cases, (**12 of which had an RPD > 30%),** the ELISA result was **greater** than the sum of the six individual microcystin results from EPA 544.
- In all except one of these 19 instances, the ELISA was also greater than the sum of the individual microcystin results from the direct inject analysis.
- This indicated that there may be additional microcystin congeners in these samples that are not being detected by EPA 544.

ELISA-EPA 544-Direct Inject Comparison



The Expansion

- Seven microcystin congeners standards that were not originally in EPA 544 were obtained.
- Individual solutions of the new compounds were run by flow injection analysis to optimize MRM (multiple reaction monitoring) transitions.
- New, optimized transitions were added to NEORSD existing EPA 544 LC/MS/MS method.
- Individual standards were run on the C8 column to determine retention times.
- Nine calibration levels that included all 13 analytes were run to determine linearity.
- Resulting curves IDP/IDA were run with a mix of all 13 microcystins and nodularin. All passed method criteria.
- MRL was established at 0.02 ppb for a 100-fold sample concentration.

The Expansion

- Analytes added

Analyte Name	Quant MRM	Calibration Range	Retention Time
Microcystin-HtyR	530.40>135.10	0.25-100 ppb	11.777
[D-Asp3]Microcystin-RR	512.80>135.10	0.25-100 ppb	12.021
Microcystin-WR	534.80>135.10	0.25-100 ppb	12.553
[Dha7]Microcystin-LR	491.45>135.10	0.50-100 ppb	12.721
Microcystin-HilR	505.30>135.05	0.25-100 ppb	12.761
[D-Asp3]Microcystin-LR	491.45>135.10	0.50-100 ppb	12.961
Microcystin-LW	1025.30>135.10	0.50-100 ppb	14.190

The Expansion – the Lot

Analyte Name	Quant MRM	Calibration Range	Retention time
Nodularin	825.20>135.10	0.25-100 ppb	11.764
Microcystin-YR	523.30>135.05	0.25-100 ppb	11.765
Microcystin-HtyR	530.40>135.10	0.25-100 ppb	11.777
Microcystin-RR	519.80>135.00	0.25-100 ppb	11.989
[D-Asp3]Microcystin-RR	512.80>135.10	0.25-100 ppb	12.021
Microcystin-LR	498.40>135.15	0.25-100 ppb	12.255
Microcystin-WR	534.80>135.10	0.25-100 ppb	12.553
[Dha7]Microcystin-LR	491.45>135.10	0.50-100 ppb	12.721
Microcystin-HilR	505.30>135.05	0.25-100 ppb	12.761
[D-Asp3]Microcystin-LR	491.45>135.10	0.50-100 ppb	12.961
Microcystin-LA	910.30>776.45	0.25-100 ppb	13.209
Microcystin-LY	1002.30>135.10	0.50-100 ppb	13.244
Microcystin-LW	1025.30>135.10	0.50-100 ppb	14.190
Microcystin-LF	986.40>135.10	0.50-100 ppb	14.805
Surrogate	514.90>135.25	NA	14.999

Expansion Validation

- IDP/IDA Results

IDP/IDA Study		MC-RR	MC-YR	MC-LR	MC-LA	MC-LY	MC-LW	MC-LF	Nodularin	MC-HtyR	MC-Dasp3RR	MC-WR	MC-Dha7LR	MC-HilR	MC-Dasp3LR	Surrogate
Replicate	1	55.708	54.234	53.762	55.835	55.007	44.319	52.089	54.618	52.740	60.517	51.403	50.416	55.308	51.757	89
	2	51.670	50.250	50.389	50.306	49.169	44.313	51.534	52.419	48.391	58.433	45.720	47.275	48.616	47.808	94
	3	50.979	49.666	47.432	51.193	52.442	46.004	50.741	50.836	47.171	56.415	44.014	47.520	48.642	46.855	90
	4	52.679	50.912	50.620	49.962	51.419	42.979	50.838	51.705	48.522	57.168	48.963	47.556	48.981	48.048	92
STD Concentration, ppb		50.000	50.000	50.000	50.000	50.000	50.000	50.000	50.000	50.000	50.000	50.000	50.000	50.000	50.000	100%
Average		52.759	51.266	50.551	51.824	52.009	44.404	51.301	52.395	49.206	58.133	47.525	48.192	50.387	48.617	
Average % Recovery		105.5	102.5	101.1	103.6	104.0	88.8	102.6	104.8	98.4	116.3	95.1	96.4	100.8	97.2	Pass ± 30%
Standard Deviation		2.086	2.043	2.586	2.724	2.421	1.239	0.633	1.617	2.433	1.794	3.301	1.488	3.285	2.156	
%RSD		3.954	3.986	5.117	5.256	4.656	2.791	1.234	3.087	4.945	3.086	6.946	3.088	6.520	4.434	Pass < 30%

Expansion Validation

- MRL/MDL Results

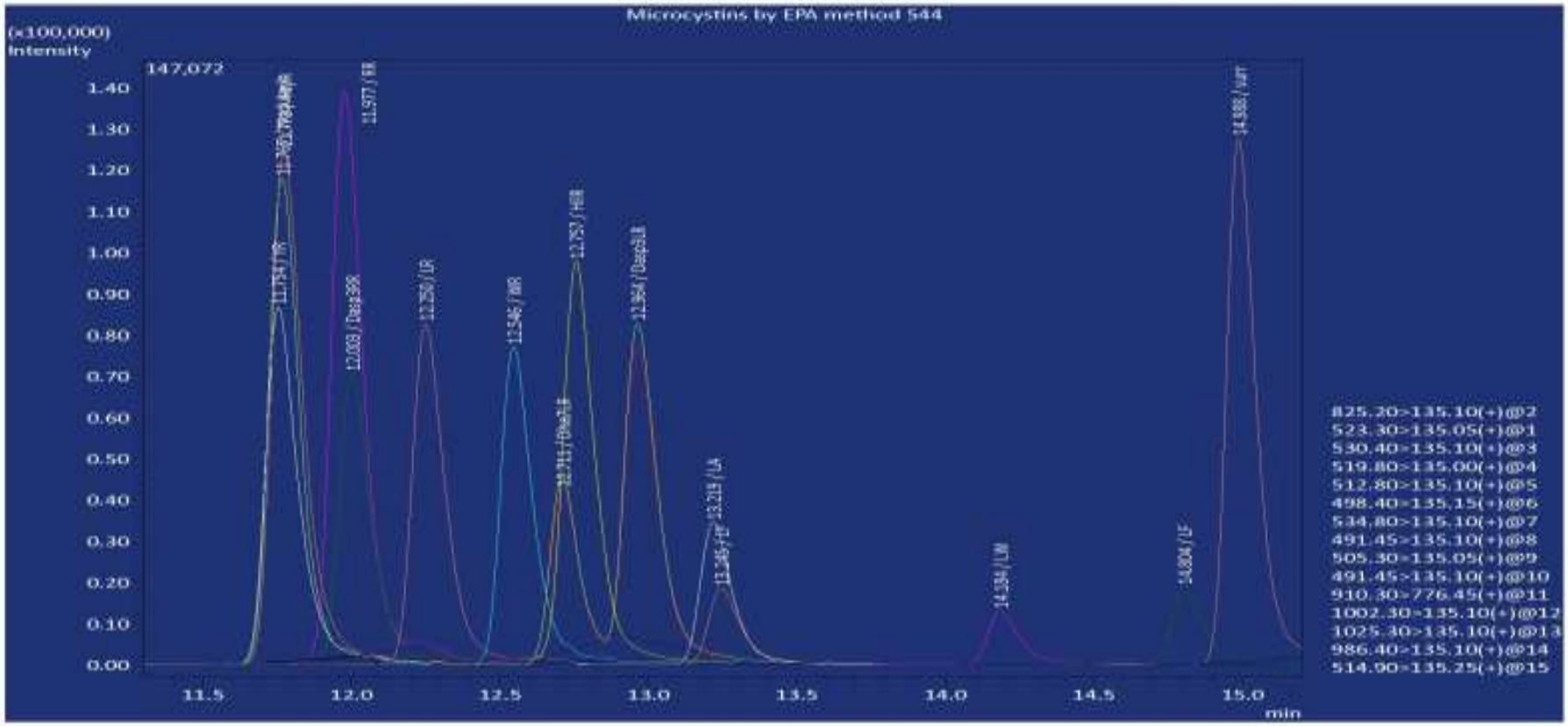
MRL/MDL Study	MC-RR	MC-YR	MC-LR	MC-LA	MC-LY	MC-LW	MC-LF	Nodularin	MC-HtyR	MC-Dasp3RR	MC-WR	MC-Dha7LR	MC-HilR	MC-Dasp3LR	Surrogate
Replicate 1	2.472	1.794	2.291	2.096	2.357	1.492	1.898	2.243	1.976	2.398	2.149	1.485	1.907	2.240	87
2	2.363	2.062	1.952	2.042	2.535	1.911	1.620	2.110	2.052	2.237	2.287	1.704	2.323	2.482	88
3	2.212	2.224	2.381	2.072	2.242	1.901	2.136	2.212	2.013	2.322	1.966	1.629	2.015	2.090	87
4	2.264	1.873	2.354	2.243	2.291	1.854	2.125	2.153	2.108	2.301	2.022	2.043	2.084	2.445	92
5	2.340	2.107	1.863	2.257	2.344	2.003	2.368	2.528	2.251	2.226	2.049	1.902	2.009	2.256	88
6	2.133	2.274	2.167	2.219	2.569	1.883	2.171	2.072	2.120	2.157	2.257	1.840	2.005	2.051	89
7	2.392	1.911	2.075	2.181	2.251	2.129	2.041	2.171	2.046	2.214	2.147	1.746	2.532	2.156	89
STD Concentration	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	100%
Student's t for n=7	3.143	3.143	3.143	3.143	3.143	3.143	3.143	3.143	3.143	3.143	3.143	3.143	3.143	3.143	
Average	2.311	2.035	2.155	2.159	2.370	1.882	2.051	2.213	2.081	2.265	2.125	1.764	2.125	2.246	
Average % Recovery	115.5	101.8	107.7	107.9	118.5	94.1	102.6	110.6	104.0	113.3	106.3	88.2	106.3	112.3	
Standard Deviation	0.115	0.182	0.201	0.088	0.132	0.196	0.237	0.151	0.090	0.080	0.120	0.184	0.221	0.166	
MDL	0.362	0.572	0.631	0.275	0.415	0.615	0.745	0.473	0.284	0.253	0.377	0.578	0.696	0.523	
PQL	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	2.000	
HRpir	0.457	0.721	0.796	0.347	0.523	0.775	0.939	0.597	0.358	0.319	0.476	0.728	0.877	0.659	
Upper PIR	138	138	148	125	145	133	150	140	122	129	130	125	150	145	≤ 150%
Lower PIR	93	66	68	91	92	55	56	81	86	97	82	52	62	79	≥ 50%

The Expansion

- Reran the beach extracts from 2015.
  - *12 of the 19 with RPDs > 30%*
  - *NOTE: A couple months out of hold time*
  - *Results of original congeners showed a small amount of degradation.*
  - *New congeners low by inference.*
- Results = original congeners results + new congener results
- Sum of the results with the 13 congeners were all greater than the original results obtained with the 6 congeners, albeit not significantly
- None of these 12 results was greater than ELISA.
- BUT, these results are indeed positive and encourage further investigation and analysis.

The Expansion

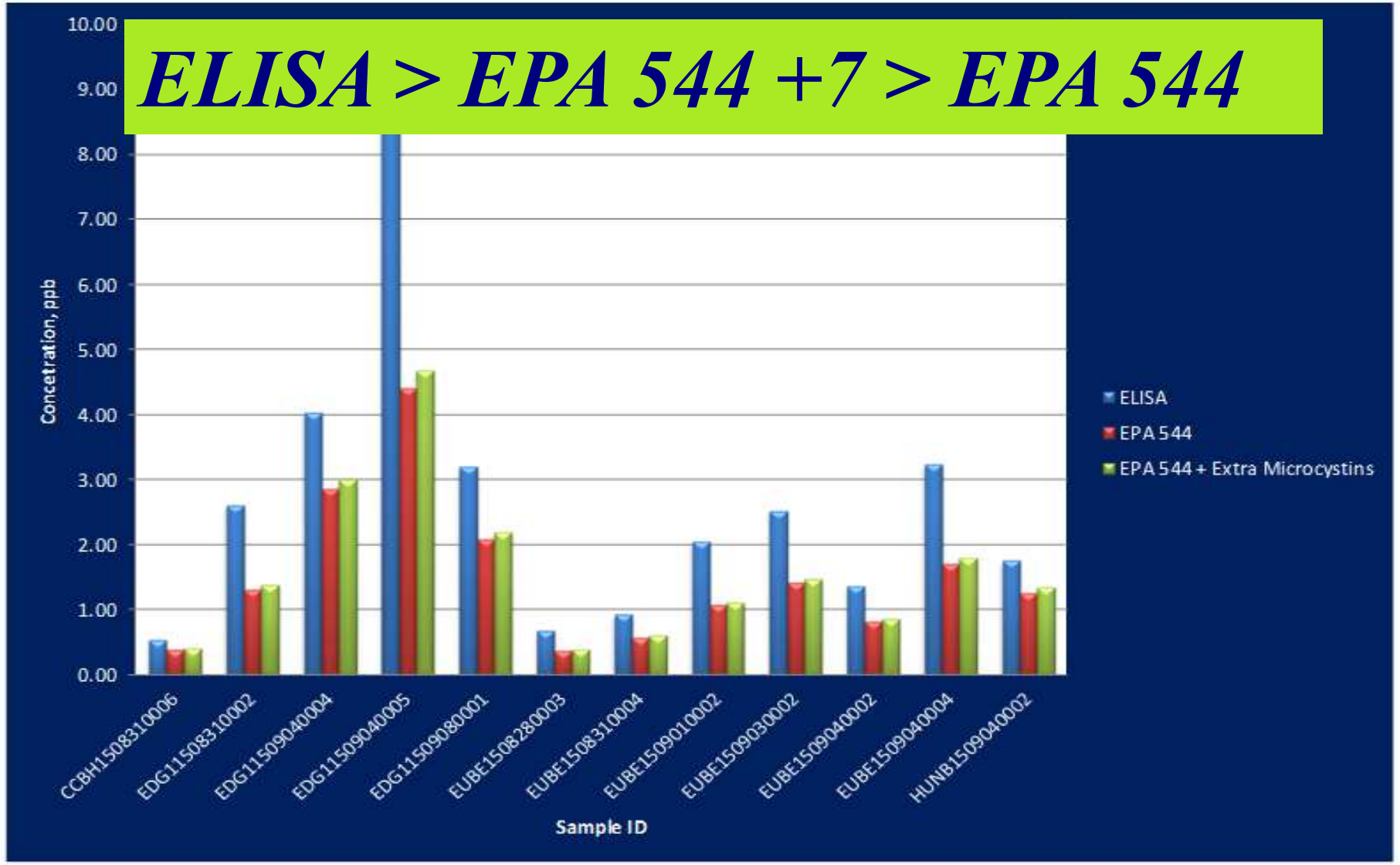
- *Typical chromatogram for a calibration standard with the 13 microcystin congeners.*



The Expansion

NEORSD Sample ID	Total Microcystins ELISA, ppb	Total EPA 544 + new Microcystins ppb	Difference ppb
CCBH1508310006	0.54	0.41	0.13
EDG1150831 0002	2.61	1.38	1.23
EDG1150904 0004	4.04	3.00	1.04
EDG1150904 0005	8.80	4.69	4.11
EDG1150908 0001	3.20	2.19	1.01
EUBE1508280003	0.68	0.40	0.28
EUBE1508310004	0.93	0.61	0.32
EUBE1509010002	2.05	1.11	0.94
EUBE1509030002	2.52	1.48	1.04
EUBE1509040002	1.36	0.86	0.51
EUBE1509040004	3.24	1.80	1.44
HUNB1509040002	1.76	1.35	0.42

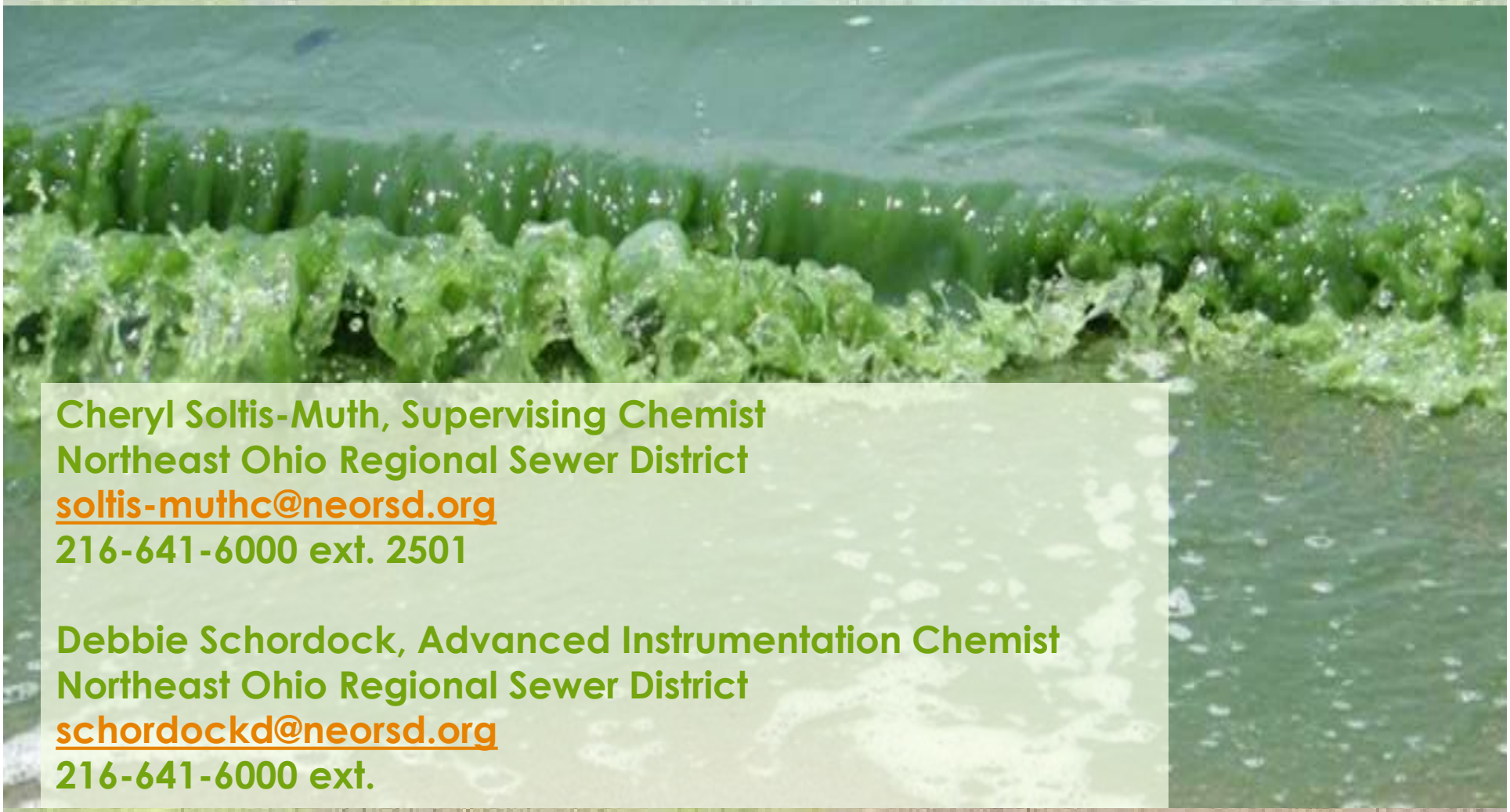
ELISA-EPA 544-EPA 544 + 7 Comparison



Future Work

- Drinking water - raw and finished data set
- Beach data set continuation from 2015
- Addition of additional congeners as standards become available
- Analysis of live cultures of toxin producing strains.
- Investigate the direct inject method for viability.
- Investigate inline SPE/concentration to reduce turn around time
- Look at the nine samples from 2015 where EPA 544 results were higher than ELISA (by > 30% RPD) including an investigation as to other causes of method variability.
- Compare LC/MS/MS results with qPCR methods being run by the lab.

Thank you for listening!



Cheryl Soltis-Muth, Supervising Chemist
Northeast Ohio Regional Sewer District
soltis-muthc@neorsd.org
216-641-6000 ext. 2501

Debbie Schordock, Advanced Instrumentation Chemist
Northeast Ohio Regional Sewer District
schordockd@neorsd.org
216-641-6000 ext.

Northeast Ohio Regional Sewer District