Cyanotoxin Analysis: Round Robin Testing and Comparison of Commercially Available Standards

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Cyanobacterial Blooms and Cyanotoxins

- **Cyanobacterial blooms**
  - Occur naturally in all bodies of water
  - Some produce toxins called cyanotoxins
  - Increased frequency and locations in recent years
  - Key factors are temperature, light, and nutrients
- **Cyanotoxins**
  - USEPA Contaminant Candidate List 4 and Unregulated Contaminant Monitoring Rule 4
  - Primary cyanotoxins
    - Microcystins (hepatotoxins)
    - Anatoxin-a (neurotoxin)
    - Cylindrospermopsin (hepatotoxin)

EPA Health Advisories for Drinking Water

	10-Day Health Advisories Issued in 2015
Microcystins (MCs)	• 0.3 µg/L for children younger than 6 years old • 1.6 µg/L for school age children through adults
Anatoxin-a (ANTX)	• None
Cylindrospermopsin (CYL)	• 0.7 µg/L for children younger than 6 years old • 3 µg/L for school age children through adults

California Voluntary Guidance for Recreational Water

	Caution	Warning	Danger
Microcystins (MCs)	0.8 µg/L	6 µg/L	20 µg/L
Anatoxin-a (ANTX)	1 µg/L	20 µg/L	90 µg/L
Cylindrospermopsin (CYL)	1 µg/L	4 µg/L	17 µg/L

- EPA issued recommended guidelines in 2019 for recreation water
  - MCs at 8 µg/L and CYL at 15 µg/L
- Other states such as Massachusetts, Minnesota, and New Jersey also have health guidelines for recreational water

Analytical Methods for Cyanotoxins

	Enzyme-linked immunosorbent assay (ELISA)	Liquid chromatography/tandem mass spectrometry (LC-MS/MS)
Characteristic	Biological – antibody response	Chemical – chromatographic peaks of specific masses
Multiple vs. single analyte	Measure toxins in groups	Measure individual variants
EPA method	Method 546 for MCs and NODs	Method 544 for MCs and NODs Method 545 for ANTX and CYL
Sample volume	<5 mL (Method 546)	500 mL (Method 544) < 5 mL (Method 545)
Reporting limit	0.3 μg/L (Method 546) 0.15 μg/L per manufacturer	0.0029 – 0.022 μg/L (Method 544) 0.018 μg/L ANTX and 0.063 μg/L CYL (Method 545)

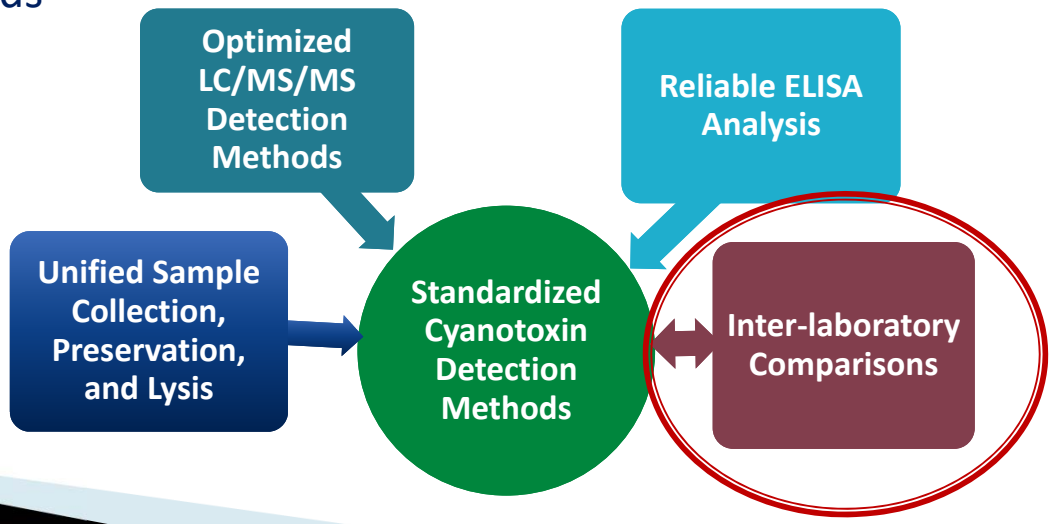
MCs, microcystins; NODs, nodularins; ANTX, anatoxin-a; CYL, cylindrospermopsin

Improvements Needed:

- ELISA
  - Cross reactivity for different MC variants
  - Results from ELISA cannot be compared directly with those from LC-MS/MS
- LC-MS/MS
  - Methods 544 and 545 by LC-MS/MS are for drinking water only
    - No EPA (or standard) LC-MS/MS methods for raw water samples
  - Different sample preservation, quenching, and lysis methods
  - Using two LC-MS/MS methods increases turnaround time and cost
  - Limited number of commercially available standards
  - Need to include more MC variants as the standards become available
  - Need a method that measures total MCs and NODs

Project 4716 Research Approaches

- Evaluation of ELISA and LC-MS/MS methods starting from sample collection, preservation, and processing
- Optimization and standardization of LC-MS/MS methods for MCs, ANTX, and CYL
- Optimization of ELISA data interpretation by using an Effect Concentration-Equivalent Concentration (EC-EQ) mass balance approach
- Inter-laboratory comparisons using methods optimized in this study as well as EPA methods



Interlaboratory Comparisons of Cyanotoxin Methods

	Round Robin 1 (RR 1)		Round Robin 2 (RR 2)	
Samples	Reagent water Surface water (no bloom) Finished drinking water		Surface water - heavy bloom Surface water - light bloom Surface water (no bloom)	
Methods	**LC-MS/MS**	**ELISA**	**LC-MS/MS**	**ELISA**
	EPA 544 EPA 545 MAC LC-MS/MS* MMPB**	EPA 546/701.0 ANTX-ELISA CYL-ELISA	MAC LC-MS/MS MMPB	EPA 546/701.0
Preservative, quenching	2-chloroacetamide, sodium thiosulfate, ascorbic acid		None	
Lysis and filtering	None		Lysis (freeze-thaw 3X), filtering	
Participating laboratories	12		12	

*MAC LC-MS/MS, a direct injection method for MCs, ANTX, and CYL

**MMPB, an LC-MS/MS method that measures total MCs and NOD after oxidation

MAC-LC/MS/MS Method

- Direct injection method for the simultaneous detection of MCs, ANTX, and CYL
- Fifteen analytes total: 12 MCs, NOD, ANTX, CYL
- Four isotopically labeled standards were used at the time of the project, with three more added since
- Reporting limits
  - ANTX and CYL 0.05 µg/L
  - MCs and NOD 0.1-0.2 µg/L
- Optimized in three laboratories on multiple instruments

QC Measures:

- ELISA kits from the same vendor and lot
- LC-MS/MS standard solutions for calibration
- Syringe filters for RR2 for sample processing
- QC samples (MCLR in Milli-Q or laboratory reagent blank from ELISA kits)

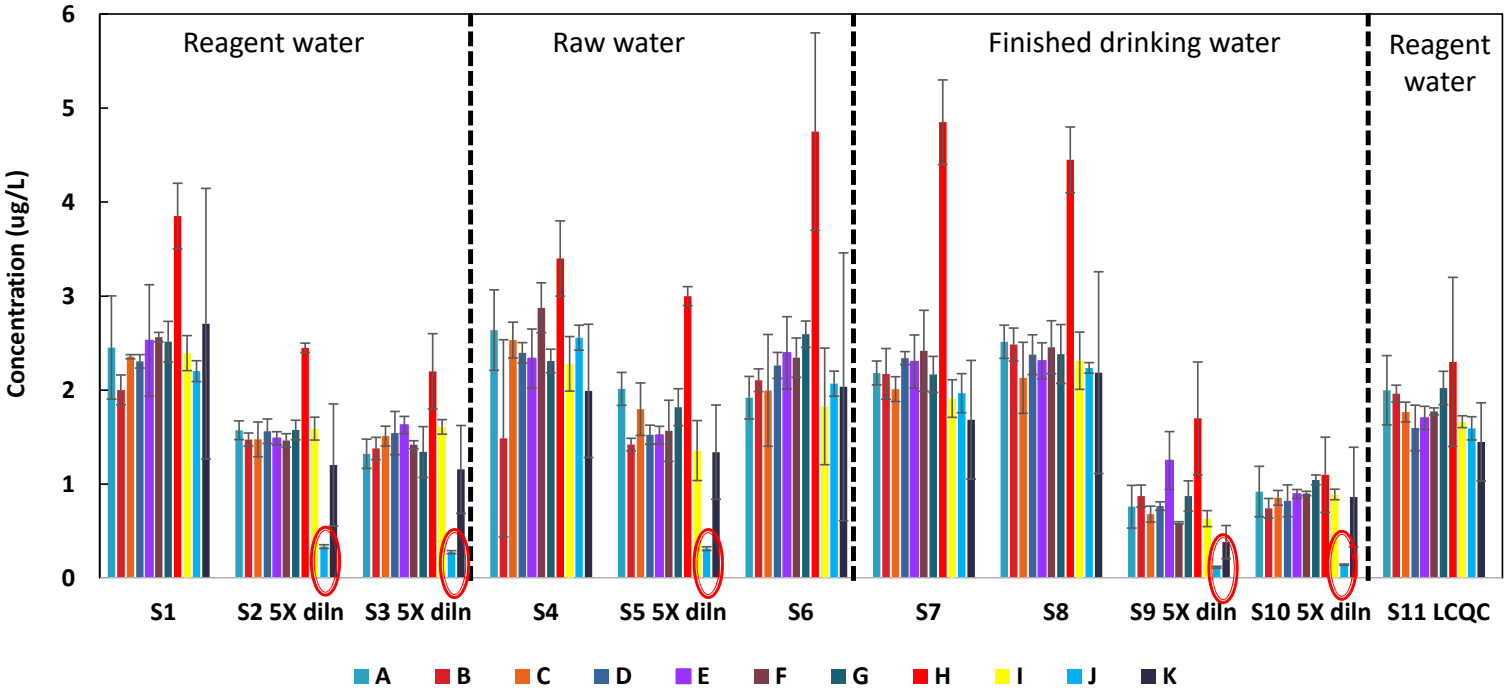
Round Robin 1 (RR 1)

Sample	Matrix	Preservative/Quenching	Spike* (MCs, µg/L)	Spike (ANTX/CYL, µg/L)
1-2**	MQ	None	0.4-0.8 ea.	0.4-0.8 ea.
3**			0.4-0.8 ea.	None
4-5**	Raw water	None	0.4-0.8 ea.	0.4-0.8 ea.
6		2-Chloroacetamide	0.4 ea.	0.4 ea.
7	Drinking water	Sodium thiosulfate	0.4 ea.	0.4 ea.
8		Ascorbic acid	0.4 ea.	0.4 ea.
9**		Sodium thiosulfate	0.8 ea.	None
10**		Ascorbic acid	0.8 ea.	None
11	MQ	None	1.0 (MCLR)	None

** RR 1 included 6 MCs from EPA 544 (MCLR, MCLY, MCLA, MCLF, MCRR, MCYR), MCWR, MCdmLR, ANTX, CYL*

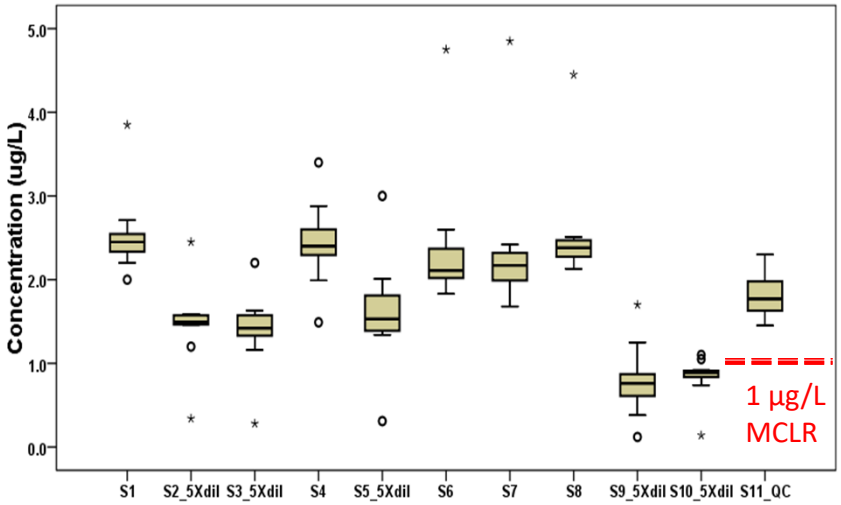
*** Samples diluted 5 times to fit in the ADDA-ELISA calibration range*

RR 1 - ELISA Data Summary



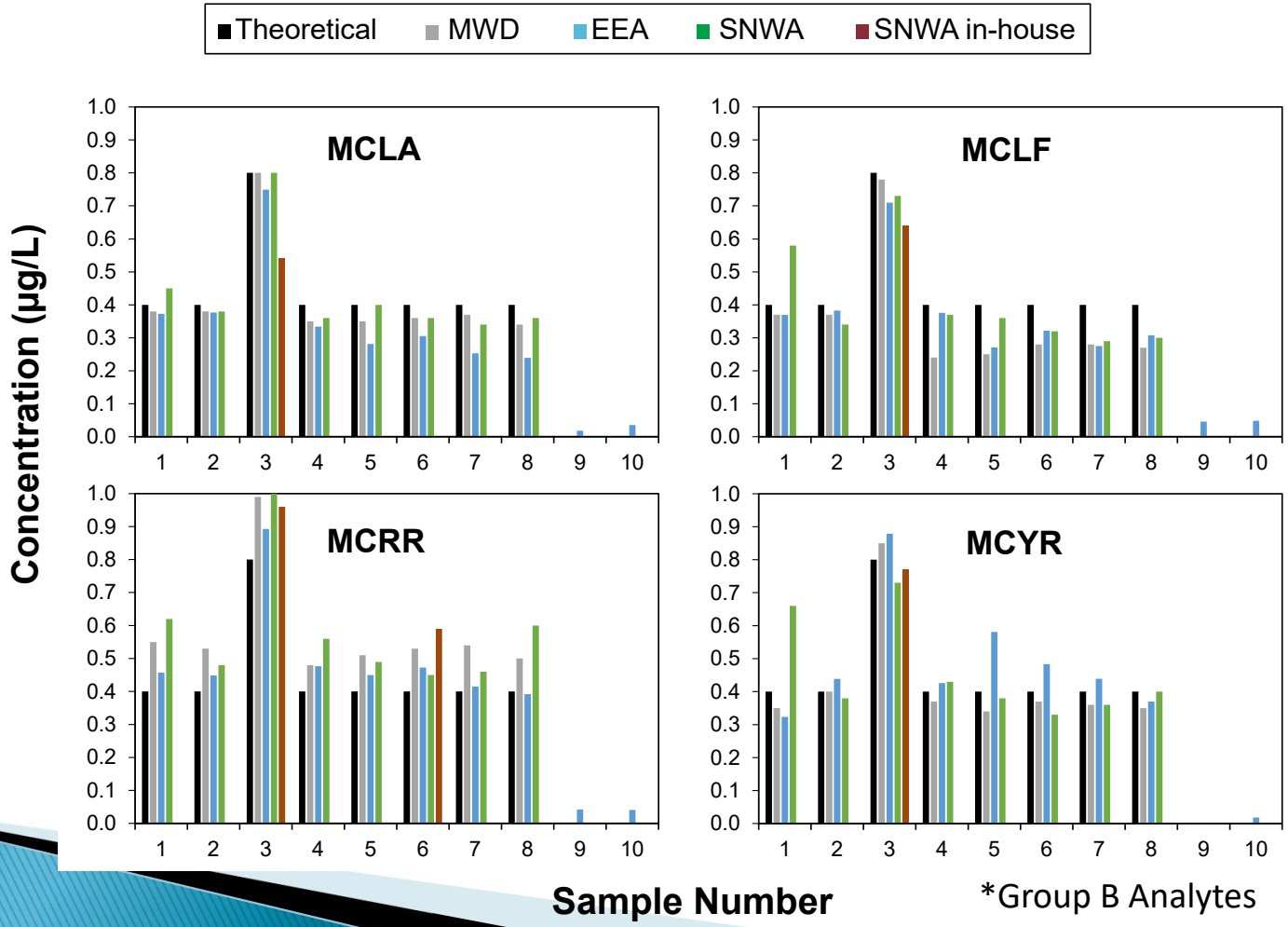
- Lab H – results biased high for all samples
- Lab J – results biased low for pre-diluted samples
- Lab K had consistent higher standard deviation than others

RR1 MC ELISA Results (cont'd)



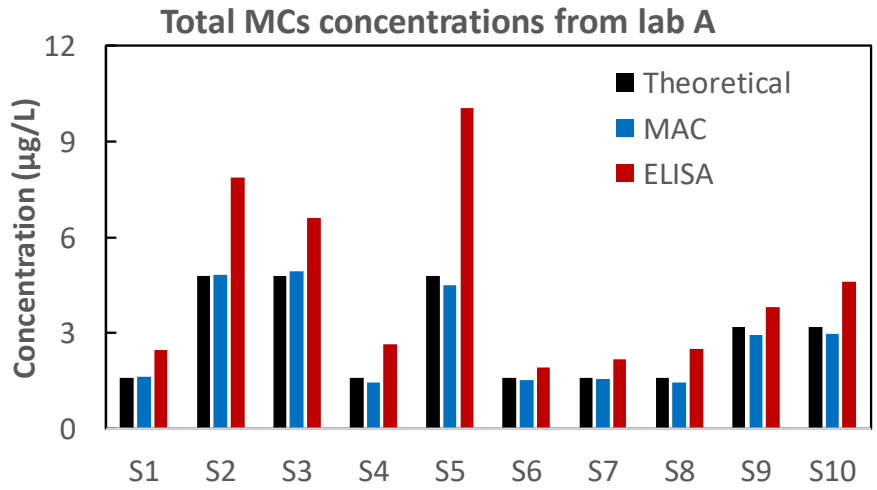
- Eleven labs used ELISA for MCs and NOD. Except for two labs, the RSDs for all samples were below 20%.
- S11 contains 1 µg/L MCLR. The average ELISA result was 1.84 µg/L, due to the concentration differences in standards between two vendors.

RR1 - MAC LC-MS/MS Results Comparison



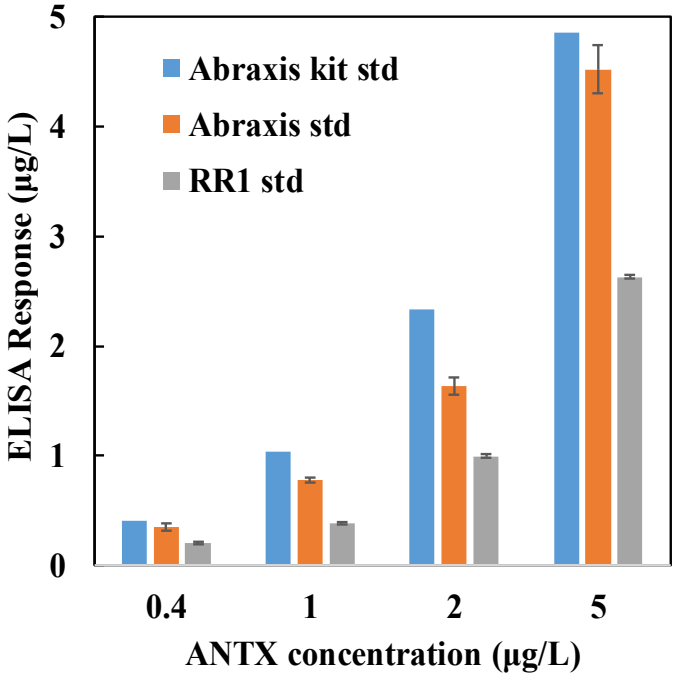
*Group B Analytes

RR1 - ELISA vs. LC-MS/MS Results



- ELISA results were 34%-79% higher than LC-MS/MS results.
- Contributing factors:
  - Standards from different vendors
  - Variants with different cross-reactivities in ELISA (e.g., MCLA and MCdmLR more reactive than MCLR)

RR1 - ANTX ELISA Results



- In contrast to LC-MS/MS, ELISA had no detections of ANTX in all samples.
- Abraxis ANTX ELISA kits respond only to (+)ANTX enantiomer.
- Standards used for spiking contained about 50% (+)ANTX, resulting in ELISA responses lower than those from LC-MS/MS.

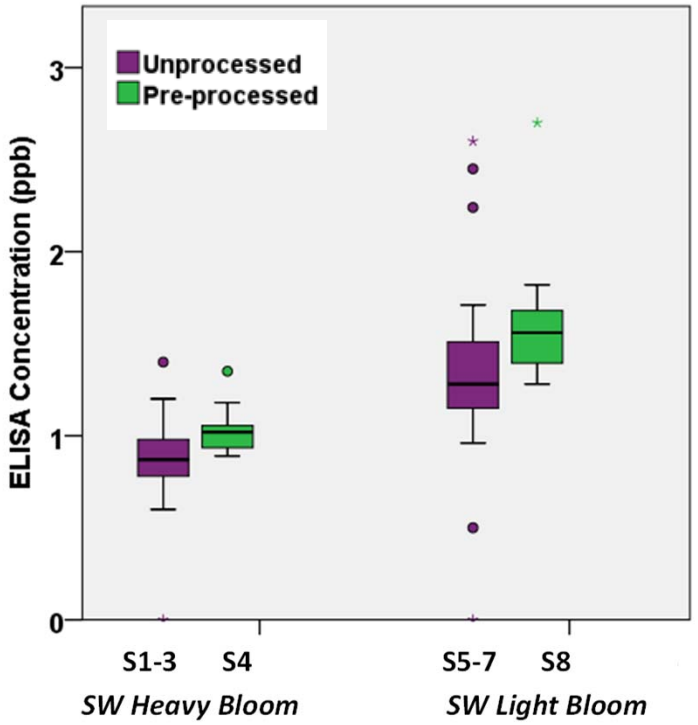
Round Robin 2 (RR 2)

Sample	Matrix	Sample Processing	Spike* (µg/L)
1-3	Surface water with a heavy bloom	Unprocessed	None
4**		Pre-processed	
5-7	Surface water with a light bloom	Unprocessed	
8		Pre-processed	
9-10**		Pre-processed	0.5 ea.
11	Drinking water treatment plant influent	Pre-processed	None
12-13**		Pre-processed	0.5 ea.
14-15	MQ; Laboratory reagent blank	Pre-processed	1.0 (MCLR)

** RR 2 included 12 MCs (MCLA, MCLF, MCLR, MCLW, MCLY, MCRR, MCWR, MCYR, MCdmLR, MCdmRR, MCHtyR, MCHilR), NOD, ANTX, CYL*

*** Samples diluted 10/50 times to fit in the ADDA-ELISA calibration range*

RR2 – ELISA Results

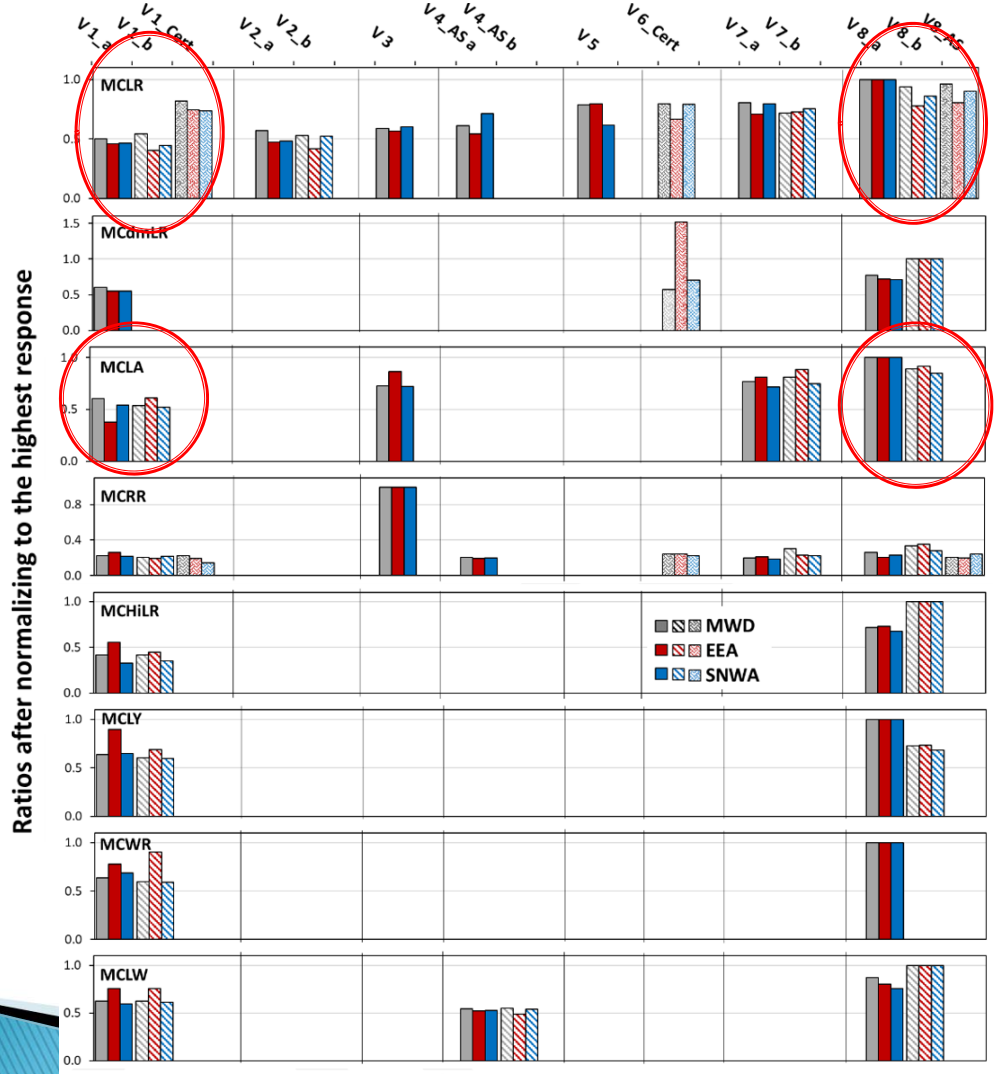


- Bloom samples pre-processed at MWD and processed at participating laboratories produced comparable results
  - Demonstrated robustness of sample preparation techniques (mixing, lysis, and filtering)
- ELISA results generally 10-30% higher than those from LC-MS/MS.

Evaluation of Concentration Variations Among Vendors, Lots, and Testing Laboratories

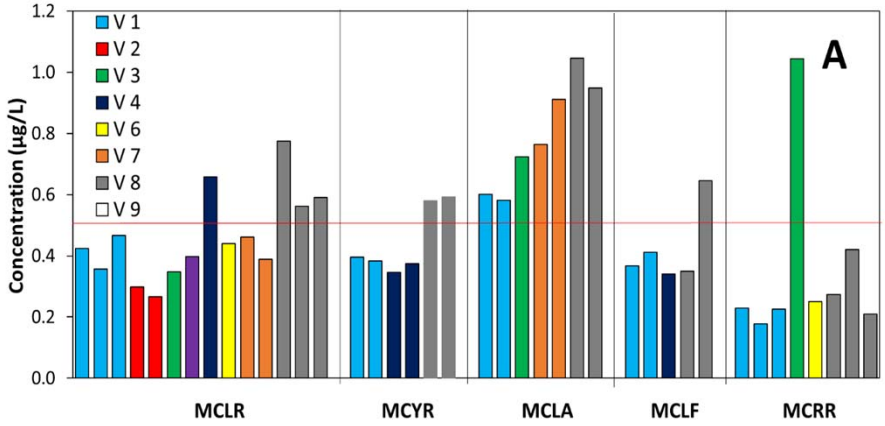
Vendor	MCLR	MCdmLR	MCLA	MCHiIR	MCRR	MCdmRR	MCLW	MCLF	MCLY	MCYR	NOD	MCWR	CYL	ANTX
V 1	3	1	2	2	3	2	2	2	2	2	2	2	3	3
V 2	2													
V 3	1		1		1								1	
V 4	1				1		2	1		2	1			
V 5	1													
V 6	1	1			1						1		1	1
V 7	2		2		2					2				
V 8	3	2	2	2	3		2	2	2	2	1	1	1	1
V 9											1			
9	14	4	7	4	11	2	6	5	4	8	6	3	6	5

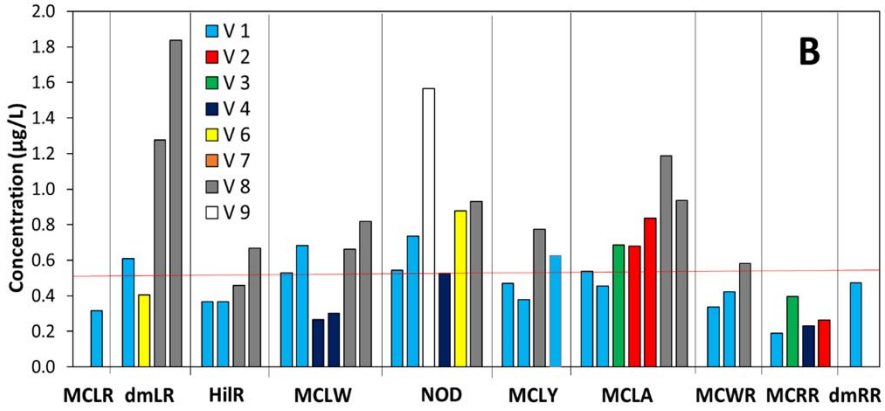
Total = 85 Standards



LC-MS/MS Results:

- Substantial differences in concentrations among vendors and sometimes between lots.
- Majority of the analytes had comparable results from three laboratories.
- Standards from V1 had the lowest responses and V8 had the highest.





ELISA Results:

- Same trends as seen with LC-MS/MS results
  - Significant concentration differences among vendors, and sometimes between lots.
  - Standards from V1 had the lowest responses and V8 had the highest.

Recommendations on Concentration Variabilities from Different Vendors:

- It is best to use standards from one vendor consistently, especially if ELISA and LC-MS/MS results are to be compared, to minimize potential concentration variations.
- It is important to keep track of lot numbers from the same vendor.
- Previous recommendations were to use UV extinction coefficients to correct standard concentrations, which the vendors can adopt and monitor to minimize the variabilities.
  - Concerns with this approach include the method not being sensitive and selective.

Conclusions

- LC-MS/MS methods:
  - MAC method and EPA Methods 544 and 545 showed good accuracy and precision, with MAC method showing the lowest standard deviations among laboratories.
- ELISA methods:
  - MC ADDA-ELISA method showed consistent results among most laboratories.
  - ANTX ELISA had no detections in RR 1 spiked samples. Standards contained both (+/-)ANTX enantiomers, however, ELISA only detects (+)ANTX.
  - MC ELISA results were higher than those from LC-MS/MS due to cross-reactivities and variabilities between standards from different vendors.
- It is best to use standards from one vendor consistently, especially if ELISA and LC-MS/MS results are to be compared.

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