U.S. Environmental Projection Agency, Office of Research and Development

SAFE AND SUSTAINABLE WATER RESOURCES RESEARCH PROGRAM

EPA Current Research on Cyanotoxins in Fish Tissue

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US Environmental Protection Agency

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Disclaimer

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Global Challenge of (HABs): treatment, detection, toxic effects, risk assessment and management.

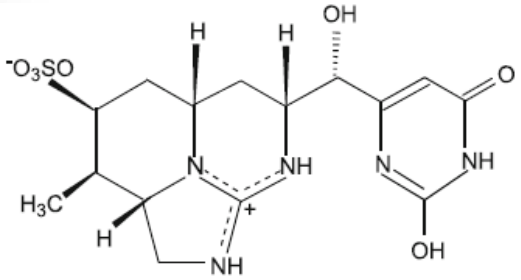
Harmful Algal Blooms (HABs) are defined as an assemblage of eukaryotic or prokaryotic plankton which have the potential to cause negative health, ecological or economic impacts.

HABs have become a recurrent, increasing and widespread issue globally, with negative impacts that include, but are not limited to, public health and environmental risks from toxin(s) production, light attenuation, diurnal swings in pH and dissolved oxygen, offensive tastes and odors, and impaired visual aesthetics.

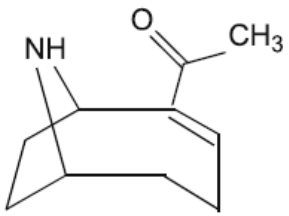
These blooms result in high cost to the water treatment and intoxication of the aquatic organisms and humans.

Studies have shown that several algal toxins can cause genotoxic effects, cellular damage and oxidative stress in fish tissues and can accumulate in the muscles, which gives the possibility of human exposure to these toxins through contaminated fish consumption.

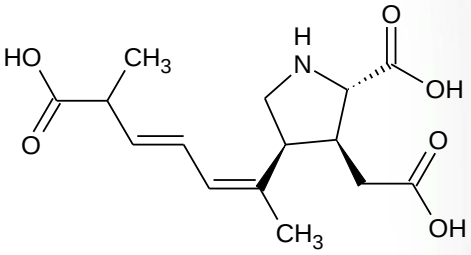
Common Cyanotoxins Associated with HABs



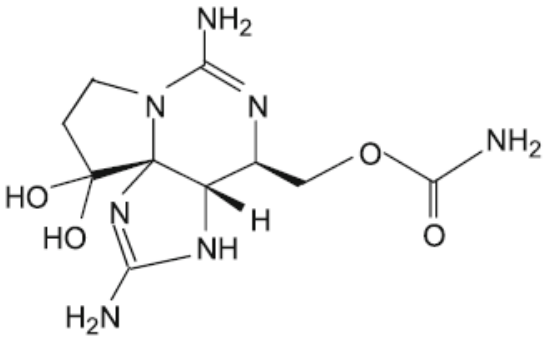
Cylindrospermopsin
Target organs: Kidney, liver



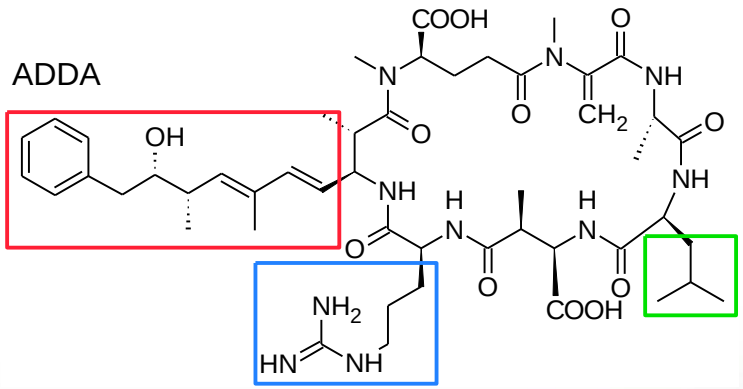
Anatoxin-A
Targets CNS



Domoic Acid
Neurotoxin/Amnesic Shellfish Poisoning



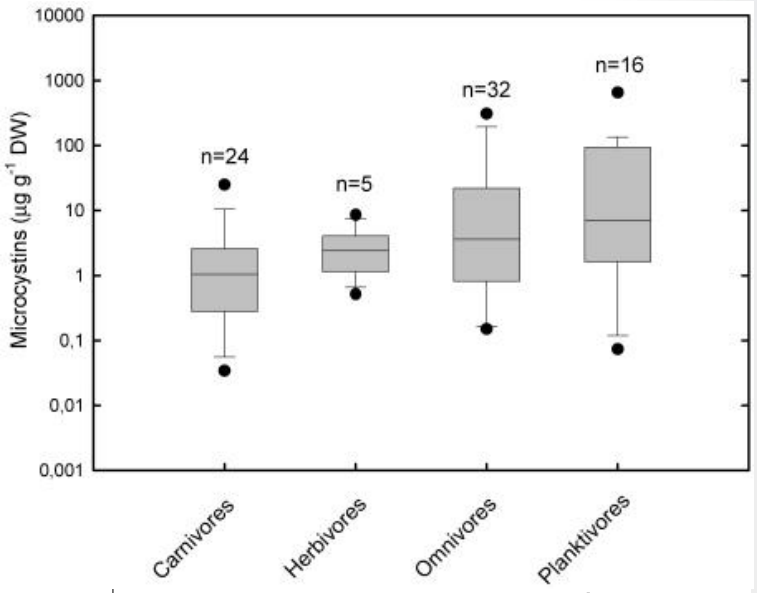
Saxitoxin
(+ Gonyautoxin, other related paralytic shellfish poisons)

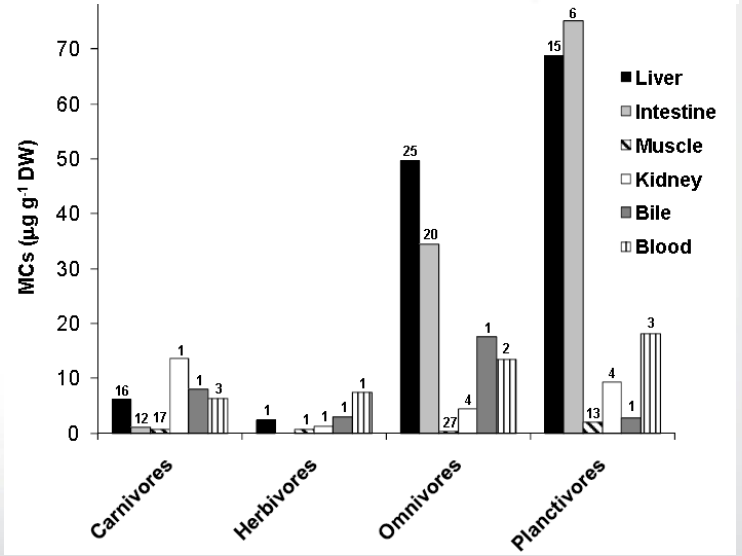


Microcystin-LR, and over 130 other congeners.
Hepatotoxic, probable carcinogen.
0.3 ug/L health advisory level in drinking water

Bioaccumulation/Biomagnification risks of Cyanotoxins

- Human health risks from consumption – where do toxins accumulate in tissue? "Are the fish safe to eat" Post-Bloom?
- Potential for bioaccumulation or biomagnification of cyanotoxins in food web
  - Bioaccumulation from consuming cyanobacteria or toxins in environment
  - Biomagnification from persistence in prey species
- Shellfish/Clams are known to bioaccumulate saxitoxins and other PSPs

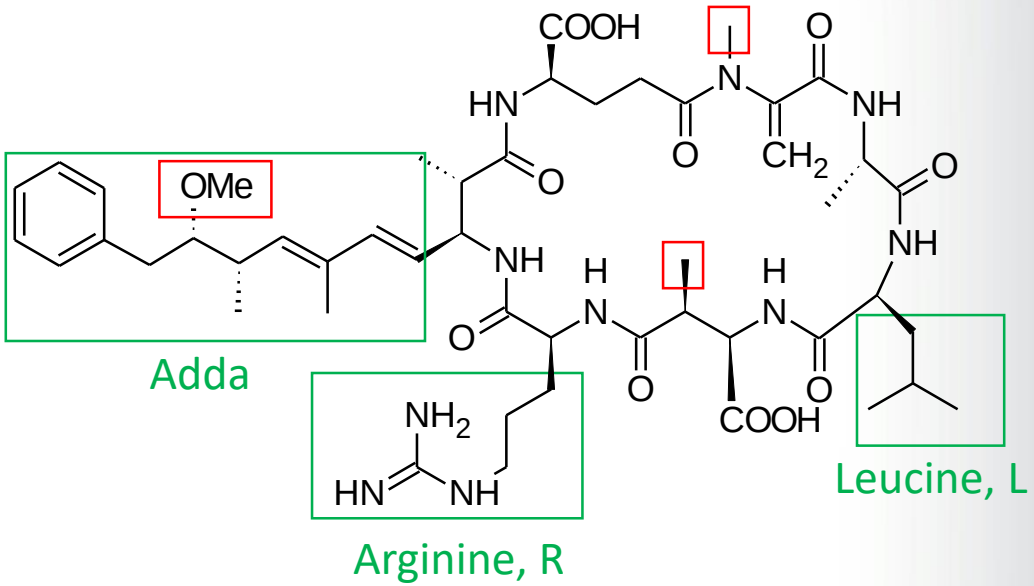




Ferrao-Filho *et al*. *Marine Drugs* **2011**, 9,2729-2772

Microcystins – An analytical challenge

- > 160 microcystin (MC) congeners have been found in the environment
  - Most common cyanotoxins in inland lakes
  - Only ~ 15 are available as analytical standards
- Variations include **amino acid substitutions** (including non-standard amino acids), methylation and desmethylation
- Chemical properties (hydrophobicity/hydrophilicity, susceptibility to treatment) can vary significantly by congener, and the congeners produced vary by species and geography



Microcystin-LR

Recovery of MC congeners from tissue

Even for "known" MC congeners there is considerable variation in recovery from tissue matrices. Unknown congeners provide an additional challenge.

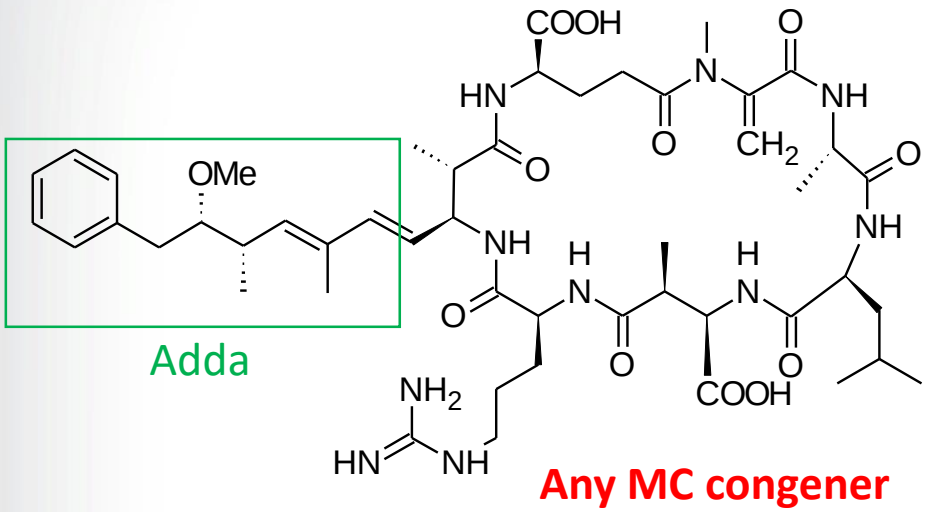
Analyte	Method 1: original QuEChERS (n=5)	Method 2: MeCN (n=5)	Method 3: MeOH (n=15)	Method 3: MeOH with filtration (n=15)	Method 4: MeCN (n=42)
MC-RR	*59*±12	*58*±1	94±*33*	86±*50*	*130*±16
Nod-R	*67*±16	*61*±13	72±18	91±17	94±10
MC-YR	82±9	74±7	*66*±17	94±19	97±17
MC-LR	90±6	*69*±14	*66*±17	89±*34*	107±15
MC-WR	79±13	*63*±8	70±17	*66*±17	115±13
MC-LA	*42*±14	84±12	*57*±9	*67*±7	90±11
MC-LY	*48*±18	84±8	*51*±13	*63*±13	91±16
MC-LW	*62*±9	*60*±10	*51*±19	*68*±*21*	107±20
MC-LF	*32*±17	*66*±16	*51*±16	*62*±*21*	104±*26*

Spike levels in catfish tissue are: method 1=100 ng/g; method 2=100 ng/g; method 3=10, 25, and 100 ng/g; and method 4=10, 25, 50, and 100 ng/g. Values in italics refer to <70% or >120% recovery and >20% SD

(Hydrophobicity generally increasing going down the series, from fillets only)

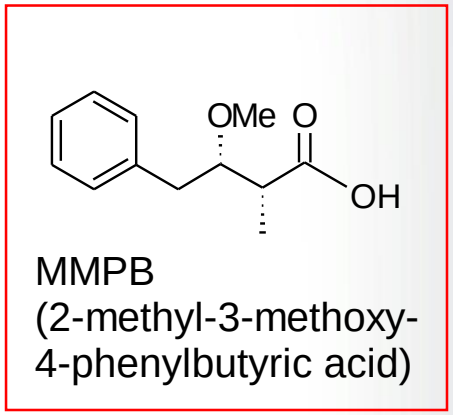
Geis-Asteggiante, L., Lehotay, S. J., Fortis, L. L., Paoli, G., Wijey, C., & Heinzen, H. (2011). Development and validation of a rapid method for microcystins in fish and comparing LC-MS/MS results with ELISA. *Analytical and bioanalytical chemistry*, *401*(8), 2617-2630.

"Total MCs" via MMPB Technique



$KMnO_4/NaIO_4$

Or Ozone, OsO_4



Application of the Lemieux Oxidation to convert the Adda moiety in all MCs present to MMPB, which is measured as a surrogate of total toxin concentration

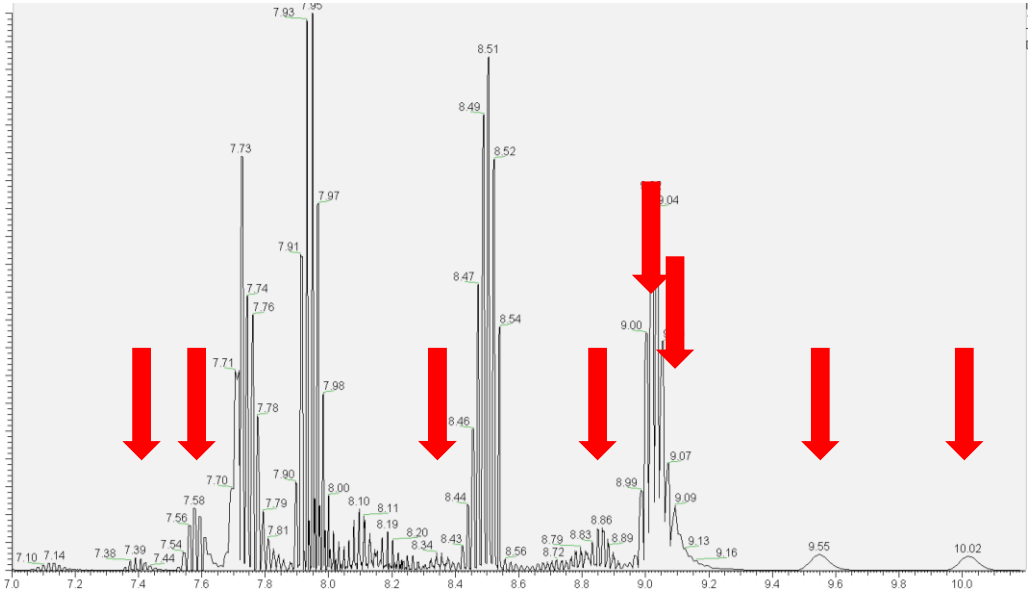
- Simplifies analysis, many congeners to one measurable product
- Cross-reactive with all microcystins containing Adda
- Simplifies extraction from complex matrices (surface water, tissue)

Lemieux, *et. al.* "Periodate-Permanganate Oxidations: I. Oxidation of Olefins", 1955, Canadian Journal of Chemistry.

Harada, *et. al.* "Mass spectrometric screening method for microcystins in cyanobacteria," *1996, Toxicon.*

Foss, *et. al.* "Using the MMPB technique to confirm microcystin concentrations in water measured by ELISA and HPLC (UV, MS, MS/MS)", Toxicon, 2015.

Why we want a “total” MC method:

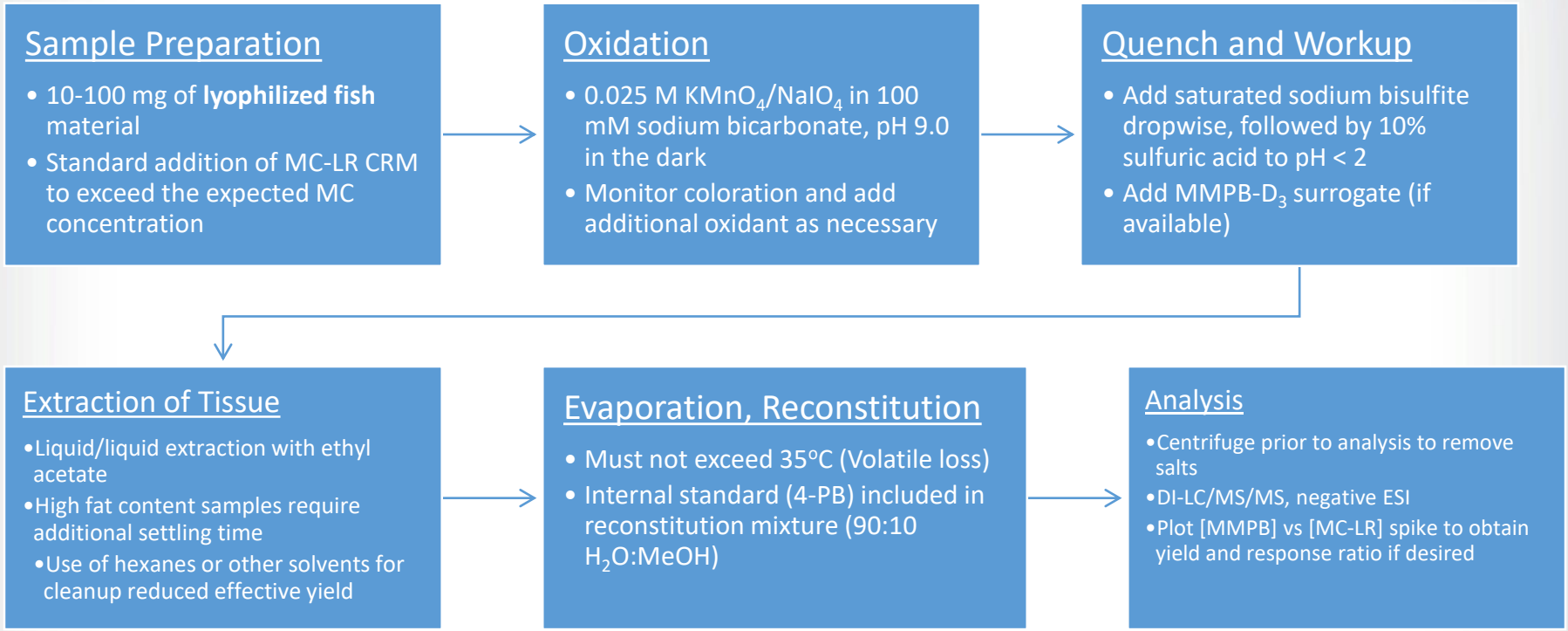


- **Surface water sample, > 32 MC Congeners observed**
  - **6 mg/L by ELISA, 5 mg/L by MMPB, 2 mg/L by LC/MS/MS with 15 congeners**
  - **Peaks in red have no analytical standards.**
- **Tissue extraction requires solvents incompatible with ELISA without solvent exchange processes, potential matrix interferences**

Study Goals

- Evaluate analyte recovery in fish tissue
  - Can we reproducibly recover MMPB?
  - Effects of lipid, species
- Spike-recovery studies in fish tissue
  - Performance with various congeners
- Application to fish in HAB-impacted water bodies
  - Sequestration of toxins to certain organs/intracellular?
- Expand to non-microcystin cyanotoxins, where direct extraction is more feasible

General MMPB Method Workflow

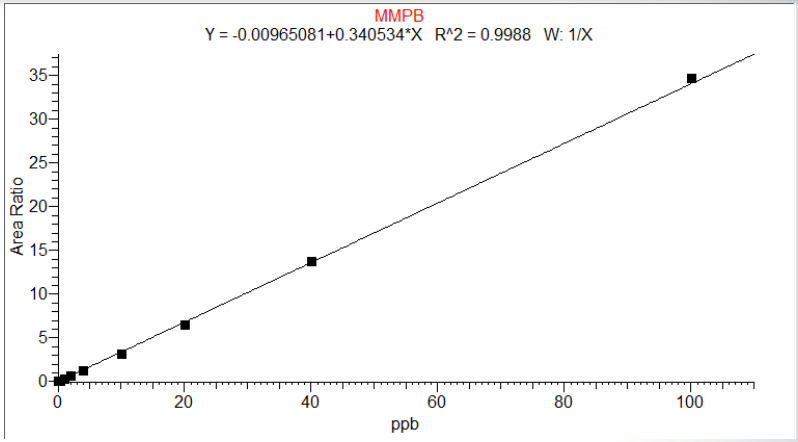


(Recovery of individual MC congeners would use a similar workflow, but omit the oxidation/quenching steps)

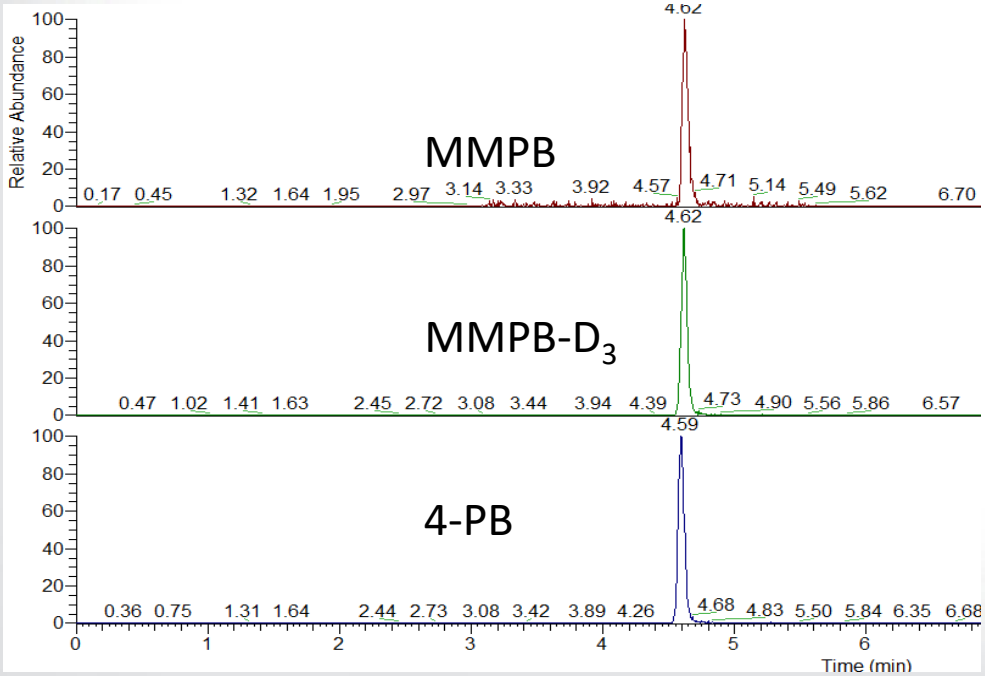
Liquid/liquid conditions similar to Sauve, *et. al.* *Analytical Chimica Acta, 2014.*

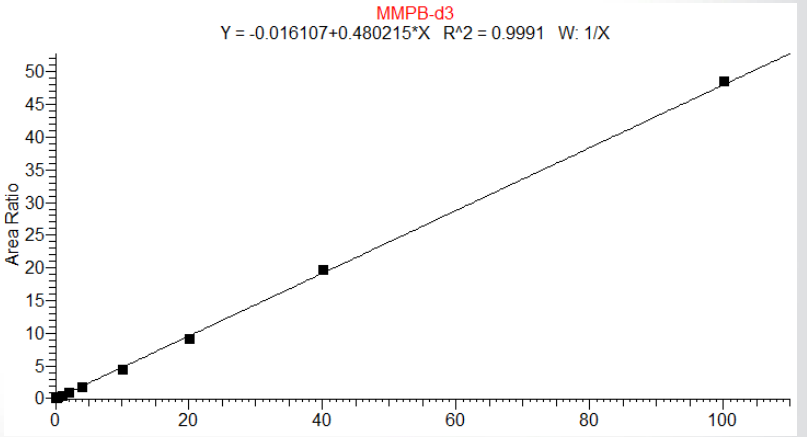
MMPB Method Analytical Details

- Reduces the analytical complexity – simple chromatography + quantitation
- Eliminates extraction variance between MC congeners
- Can quantify unknown/isomeric congeners as part of 'total' MCs



MMPB Calibration Curve, 0.1 to 100 ug/L





MMPB-D_3 Calibration Curve, 0.1 to 100 ug/L

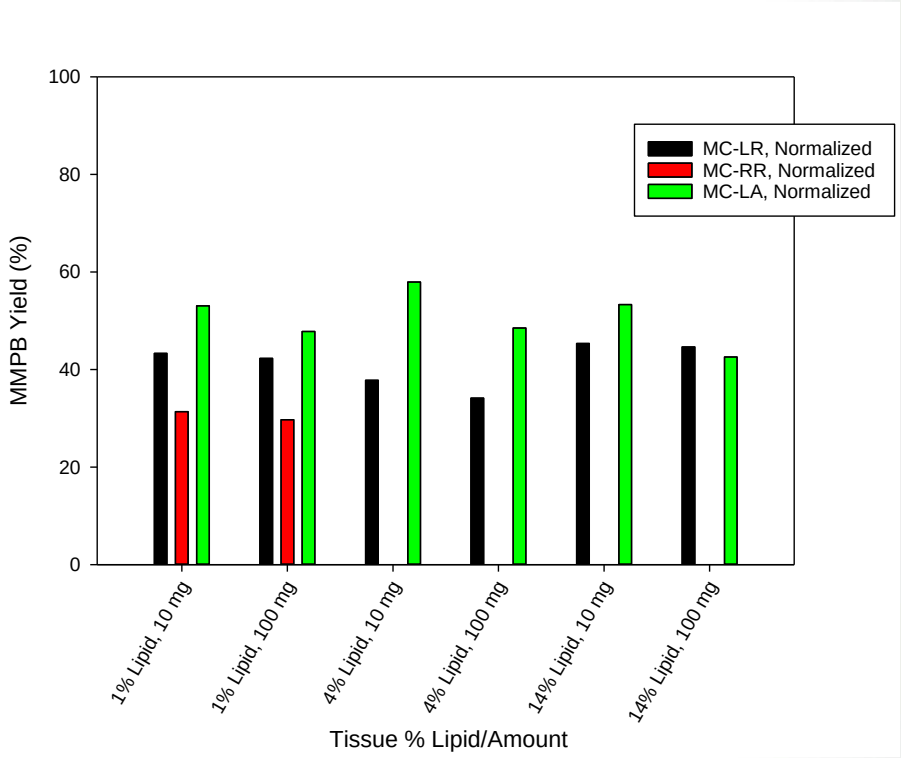
MMPB Application to Fish Tissue – MMPB Spike/Recovery Studies

- Spikes at high (40 ng) and low (4 ng) MMPB and MMPB-D_3 were performed to evaluate extraction performance.
- Consistent recovery with low and high fish samples, and for 4 and 14% lipid samples.
- Recovery of MMPB in 'blank' samples (9, 10) shows stability under derivatization conditions even in low background matrix.

Sample #	MMPB Spike (ng):	MMPB-D3 Spike (ng)	Fish (mg)	Lipid %	MMPB % Recovery
1	40	40	10	4	85
2	40	40	100	4	102
3	40	40	10	14	84
4	40	40	100	14	73
5	4	4	10	4	81
6	4	4	100	4	61
7	4	4	10	14	87
8	4	4	100	14	79
9	40	40	0	na	102
10	4	4	0	na	83

MMPB Application to Fish Tissue – Microcystin Spike/Oxidation Results

- To evaluate congener response in tissue matrices spikes were performed, followed by MMPB procedure
- Three fish matrices were tested: largemouth bass, brown trout, and channel catfish, with 1%, 4%, and 14% lipid content, respectively)
- Effects of lipid content on MMPB recovery were not significant
  - Reaction workup procedure becomes messier, but surrogate correction allows for efficiency correction



MMPB Application to Fish Tissue – MC Mixture Spike/Recovery Studies

- Mixtures of microcystins were also tested to see if hydrophobicity/hydrophilicity would influence recovery from tissues
- MMPB yields for MC-LA and MC-RR were not significantly different from 1 to 14% lipid content in the spiked tissue.
- Overall yields were typically 30-40% MMPB based on spike amounts
- Some discrepancies in standard concentration complicate 'absolute' MMPB yield (MC standards were ~50% of certified reference standards upon comparison – this is a common issue in cyanotoxin studies)

Sample:	MC-LA	MC-RR	Lipid %	Normalized MMPB Yield:
1	20	20	0	35%
2	30	10	0	39%
3	10	30	0	31%
4	20	20	1	32%
5	30	10	1	34%
6	10	30	1	33%
7	20	20	1	29%
8	30	10	1	32%
9	10	30	1	33%
10	20	20	14	37%
11	30	10	14	33%
12	10	30	14	32%
13	20	20	14	32%

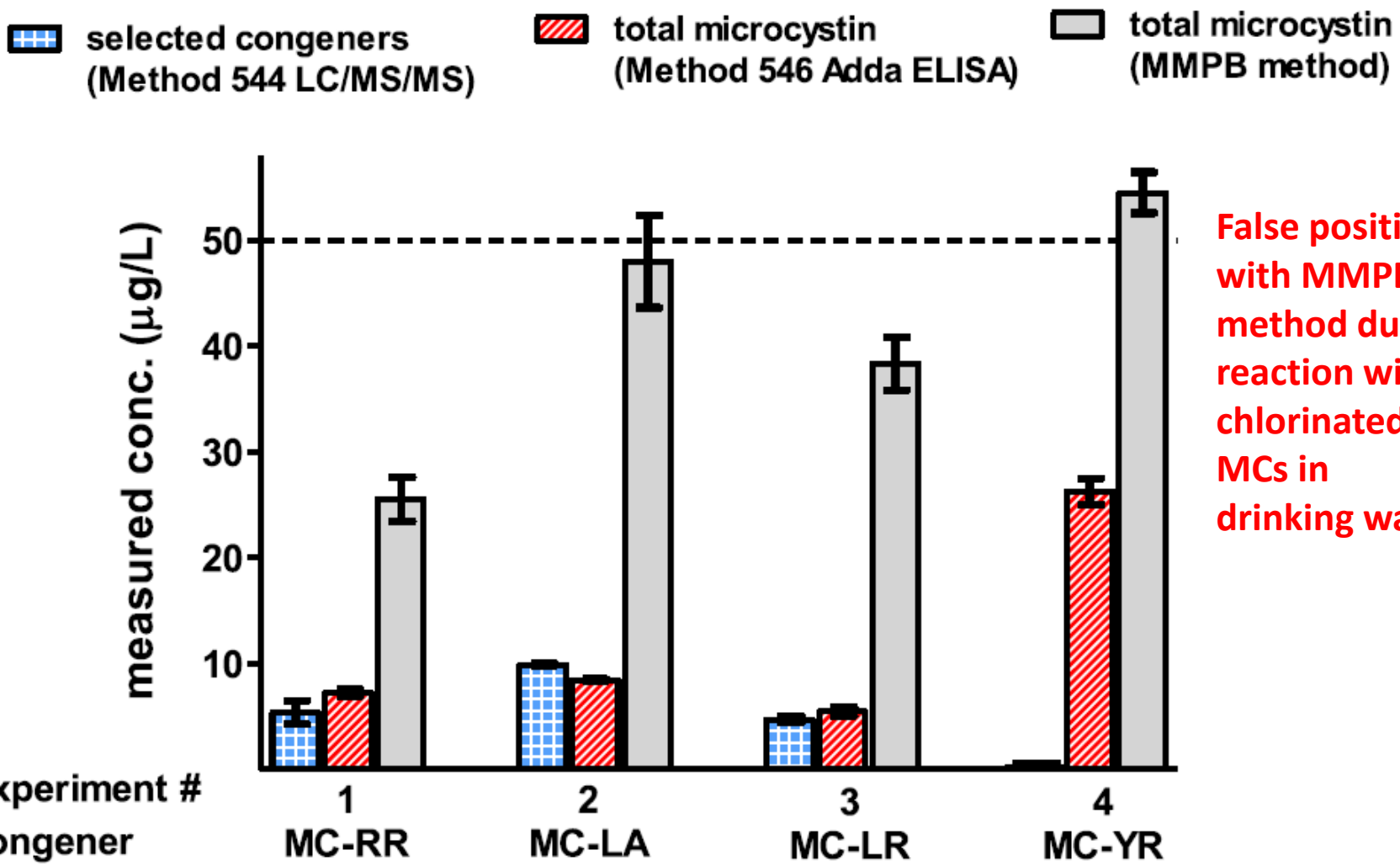
MMPB Application to Fish Tissue – Field Studies

- Presently applying the method to field studies
  - Spiking tissue may not adequately represent state of bioaccumulated toxins, particularly concentration in organs or fats
- To-date have tested carp from a fish kill in an Ohio lake (negative, possibly rotten) and fathead minnows from an on-site study where a bloom was observed (positive)
- Sample collection associated with multiple ongoing research efforts on lakes with endemic HAB activity

Sample:	Measured MMPB, ug/L	Surrogate Recovery:	Estimated Microcystins, ug/kg
Carp, 100 mg tissue	nd	86%	nd
Carp, 200 mg tissue	nd	95%	nd
Fathead Minnow, 100 mg tissue	< MRL	80%	< MRL
Fathead Minnow, 200 mg tissue	0.12	75%	15

Sample:	ug/kg MCs
Minnow 1	< MRL (< 10 ug/kg)
Minnow 2	< MRL
Minnow 3	13
Minnow 4	12
Minnow 5	< MRL
Minnow 6	29
Minnow 7	< MRL
Minnow 8	< MRL
Minnow 9	< MRL
Minnow 10	< MRL
Minnow 11	< MRL
Minnow 12	< MRL
Minnow 13	< MRL
Minnow 14	< MRL
Minnow 15	< MRL
Minnow 16	< MRL

Office of Water (USEPA) – MMPB Not Suitable for Drinking Water Monitoring

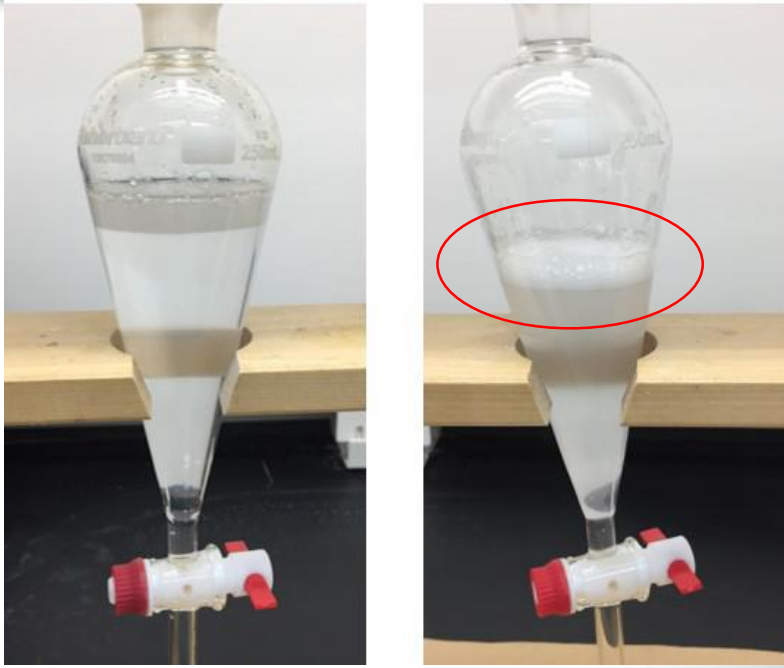


False positives with MMPB method due to reaction with chlorinated MCs in drinking water

Rosenblum, *et al. Toxicon 138 (2017), 138-144*

Conclusions

- The MMPB technique can be reliably employed for microcystin quantification in fish tissue and appears to perform well with even high lipid content
- Method quantitation limits of 0.1 to 100 ug/L MMPB correspond to roughly 1 to 1000 ug/kg MCs, depending on dilution factors/mass balance
  - For higher lipid fish samples significant impacts on sample quality are observed – primarily oils and fatty residues following sample processing
- On a per-sample basis the labor requirement is significantly higher than for ELISA or conventional LC/MS/MS analysis, as is the initial training requirement
- Field studies underway to evaluate performance
  - Compare spiking toxins to 'ambient' recovery from tissue
  - Food web implications in study water bodies?



Extraction of a 10 mg fish sample (left) and 100 mg fish sample (right)

Acknowledgements & contact information

- Acknowledgements:
  - Devi Sundaravadivelu, Jennifer Jones

- Contact:

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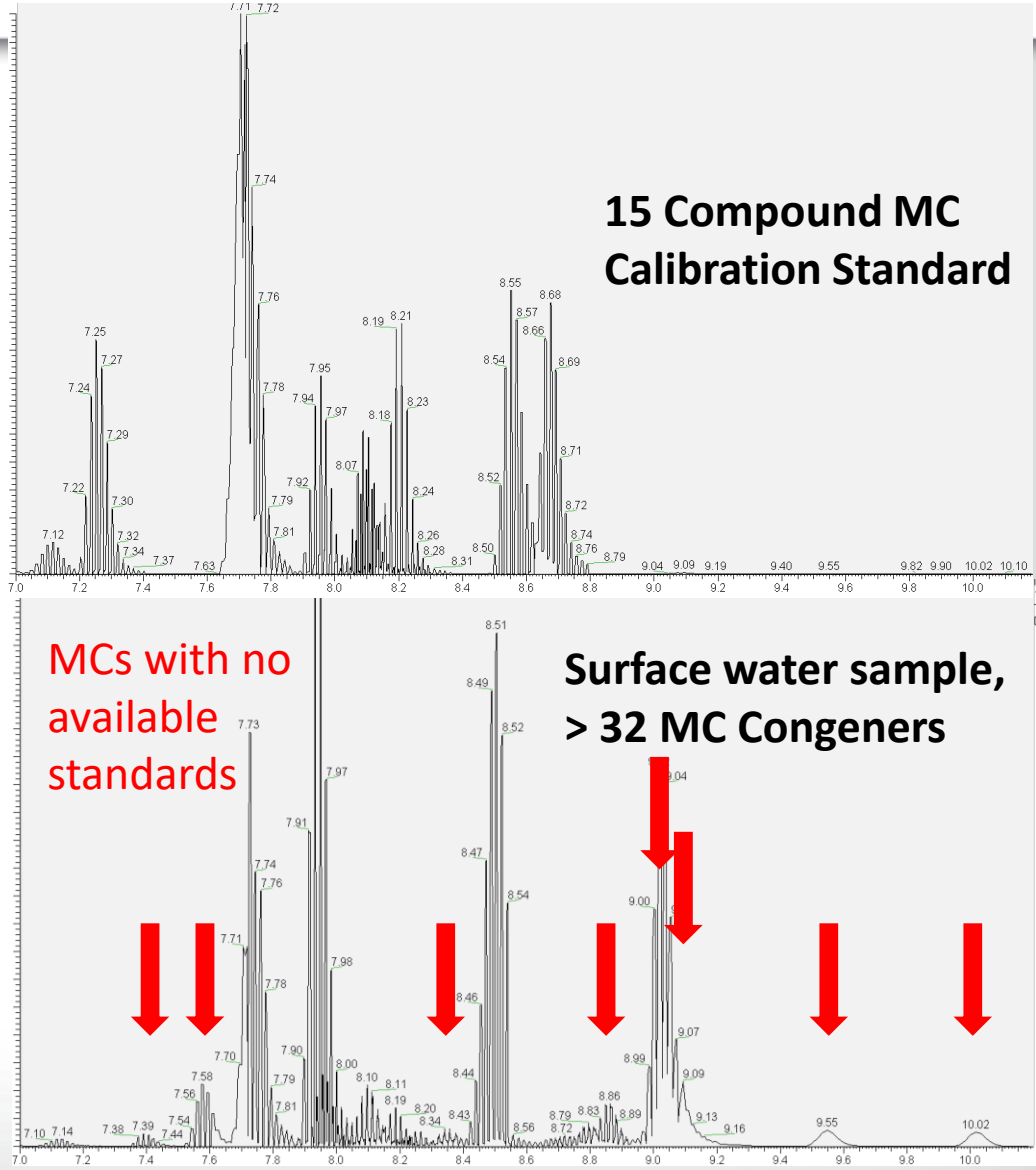
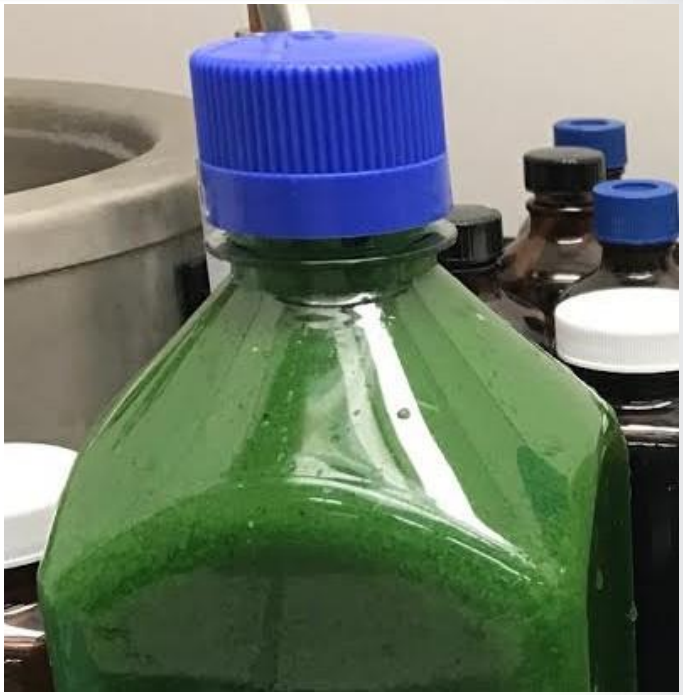
LC/MS/MS Measurement of Microcystins

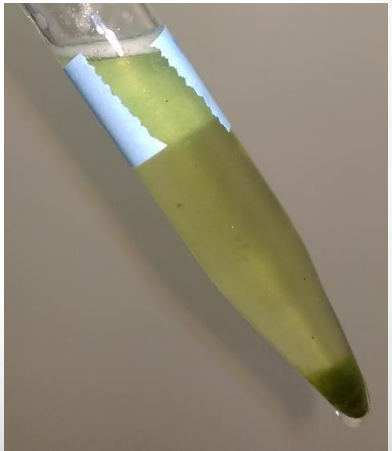
- LC/MS/MS methods will typically only measure ‘known’ congeners
- Some types of analysis can identify (but not quantify) unknown congeners containing Adda
  - LC-PDA can provide aggregate quantitation as well, but may have interferences
- Even if identified, MS-based screening for large numbers of congeners is beyond the limits of existing hardware



Application of MMPB oxidation to surface water samples

- Challenges for analyzing surface water samples for toxins include sample preparation with high biomass samples (especially scum)
- Replicate analysis also complex due to heterogeneity of samples
  - One aliquot may vary +/- 100% in sufficiently nasty samples
- Biomass is compatible with oxidation, but is *very* detrimental to the workup, particularly for scum samples
- For high biomass samples 80:20 methanol was used to disrupt cells and extract toxins, followed by centrifugation





Comparison of MMPB Conversion Yields by Matrix and Sample Preparation

Source	% Yield (Std. Addition)	[MCs] by MMPB, ug/L	[MCs] by ELISA, ug/L
Lake 1	65	940	1900
Lake 2	83	2.6	3.2
Lake 3 (Scum)	75	5120	6200
Lake 4 (Scum)	67	63000	39000
Lake 5	65	1870	1860
Lake 6	64	530	490
Lake 7	50	6.7	14.1
Lake 7 (Raw)	35	8.1	14.1
Lake 8 (Raw)	32	9.6	N/A

- Across geographically diverse lakes MMPB recoveries were generally within 50-80% for standard addition
- For comparison, samples were exposed to MMPB conditions without processing and provided comparable MC measurements, but reduced % yields