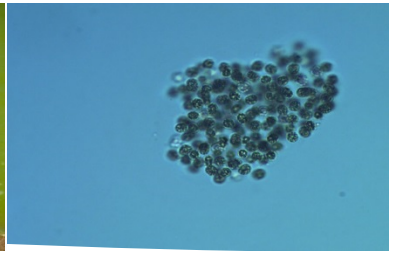
U.S. Environmental Projection Agency, Office of Research and Development

SAFE AND SUSTAINABLE WATER RESOURCES RESEARCH PROGRAM



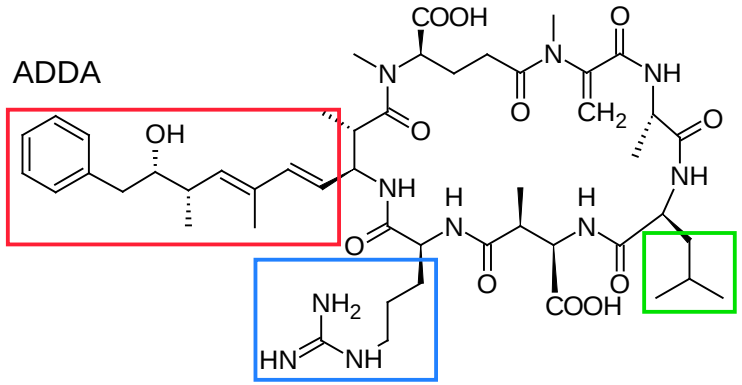

EPA Current Research on Cyanotoxins in Fish Tissue

Toby T. Sanan, Jim Lazorchak

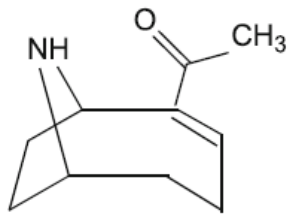
US Environmental Protection Agency

New Jersey Water Monitoring Council – Fish and Shellfish Tissue Monitoring, September 20th, 2017

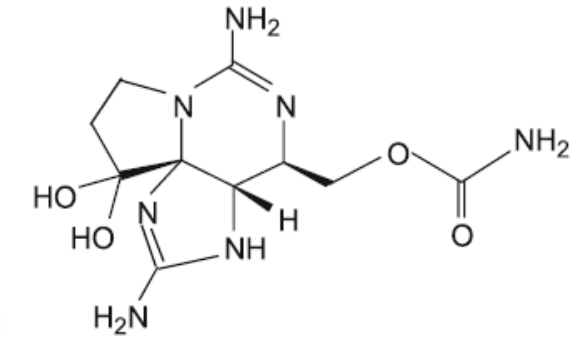
Common Cyanotoxins Associated with HABs



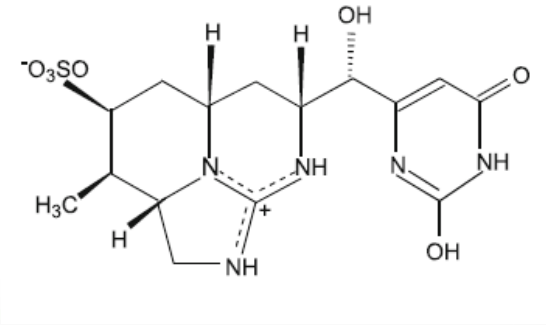
Microcystin-LR, over 130 other congeners
Hepatotoxic, probable carcinogen.
0.3 ug/L health advisory level



Anatoxin-A
Targets CNS



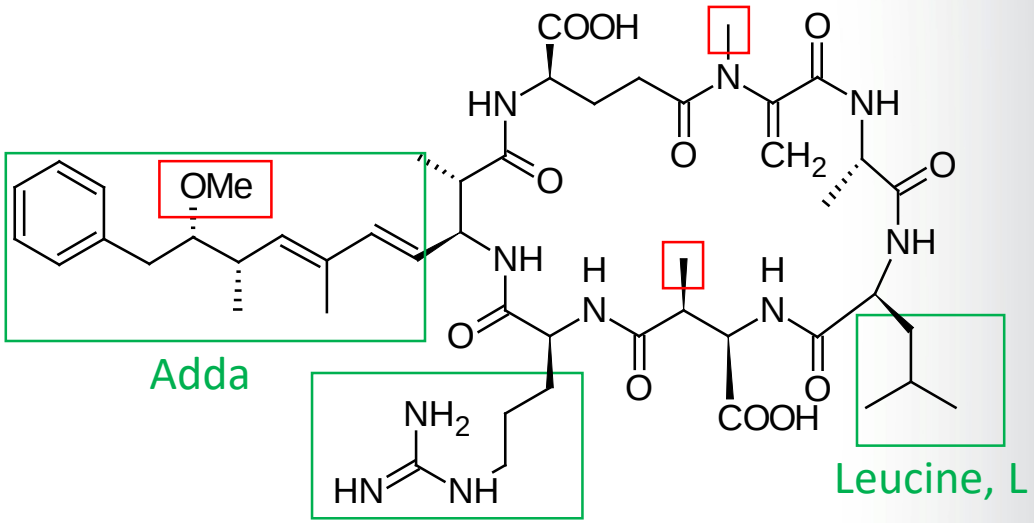
Saxitoxin



Cylindrospermopsin
Target organs: Kidney, liver

Microcystins – An analytical challenge

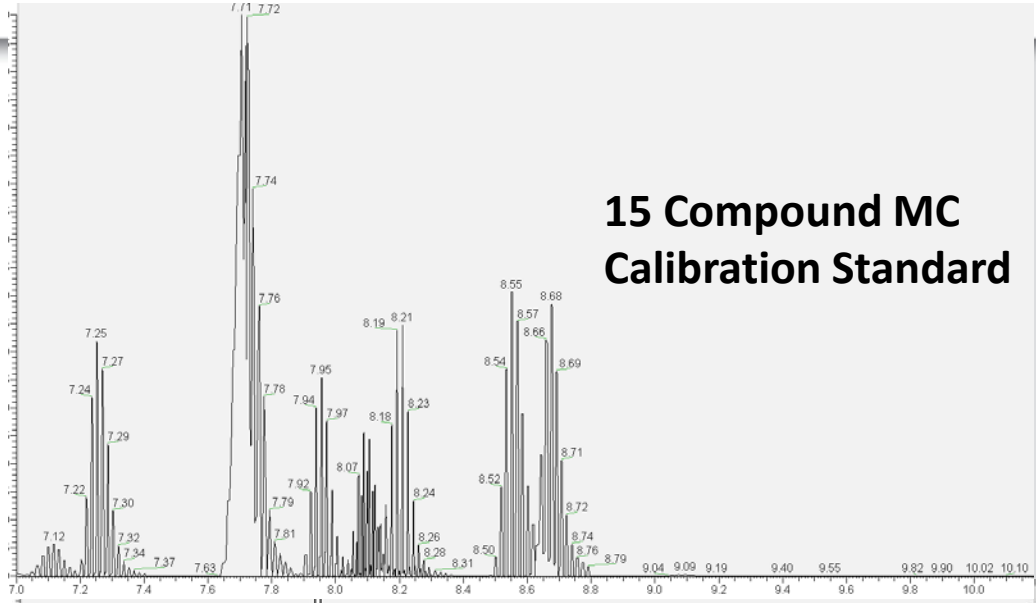
- > 160 microcystin (MC) congeners have been found in the environment
  - Only ~ 15 are available as analytical standards
  - Difficult to resolve chromatographically due to chemical similarity
- Variations include **amino acid substitutions** with non-standard amino acids
- Some congeners known to bond to tissue, others have varying extraction efficiencies

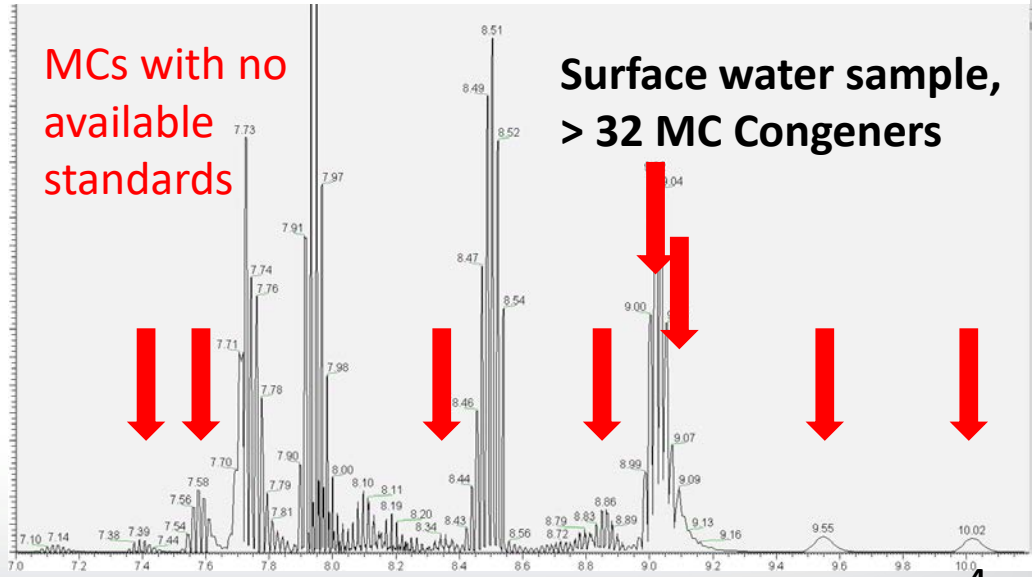


Microcystin-LR

LC/MS/MS screening tools for unknown MCs

- 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





Challenge of recovery MC congeners from tissue

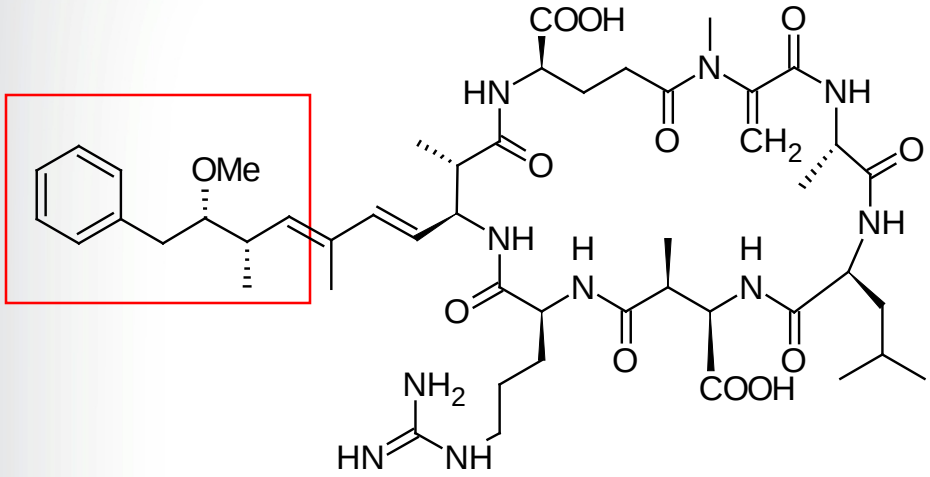
Even for “known” congeners there is considerable variation in recovery efficiency from tissue matrices. Unknown congeners provide an additional challenge.

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

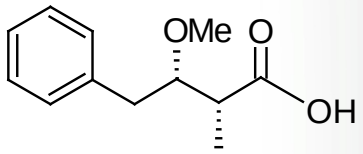
Geis-Asteggiante, L., Lehotay, S. J., Fortis, L. L., Paoli, G., Wijey, C., & Heinzen, H. (2011). Development and validation of a rapid method for microcystins in fish and comparing LC-MS/MS results with ELISA. *Analytical and bioanalytical chemistry*, *401*(8), 2617-2630.

Chemistry of the MMPB Technique



$KMnO_4/NaIO_4$

Or Ozone, OsO_4



MMPB
(2-methyl-3-methoxy-4-phenylbutyric acid)

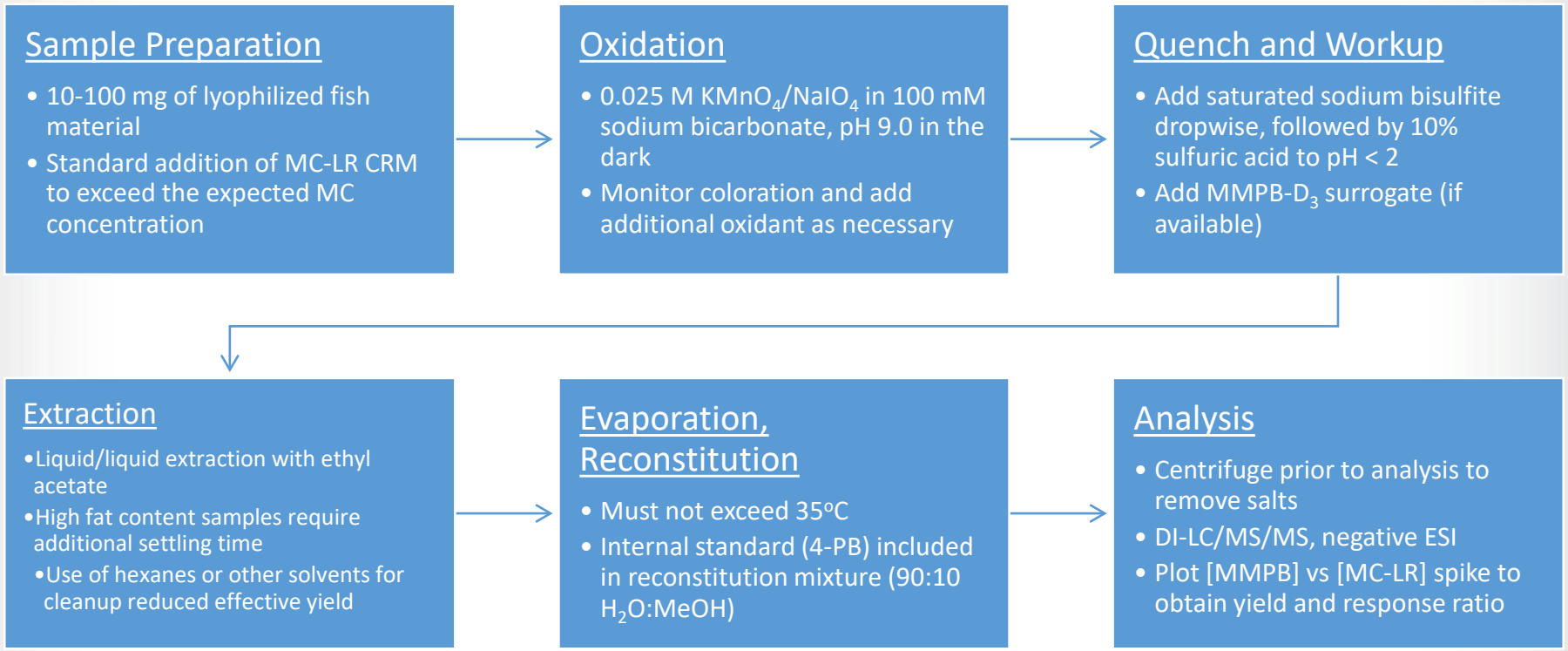
- Application of the Lemieux Oxidation to convert the Adda moiety to MMPB
  - Simplifies analysis, many congeners to one measurable product
  - Cross-reactive with all microcystins containing Adda
  - Simplifies extraction from matrices (water, tissue)

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.

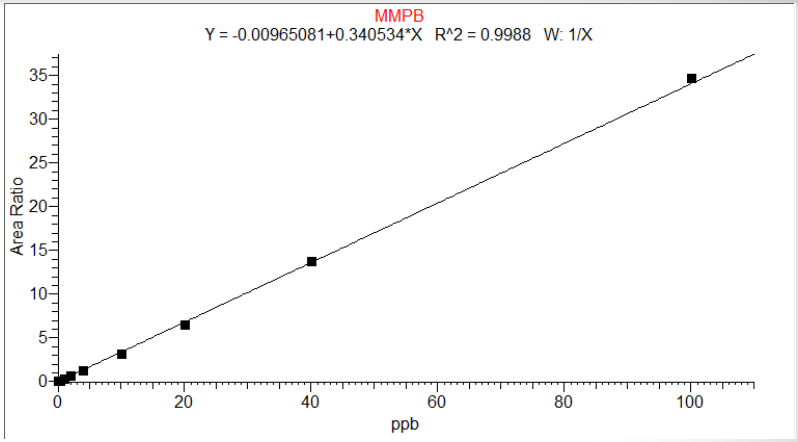
General MMPB Method Workflow



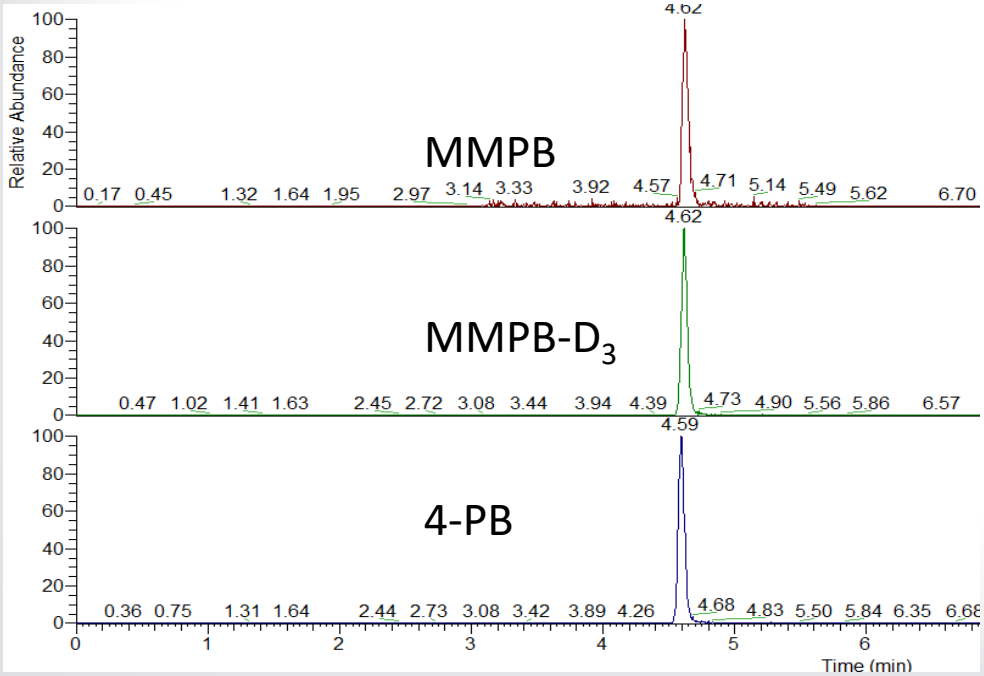
Liquid/liquid conditions similar to Sauve, *et. al. Analytical Chimica Acta, 2014.*

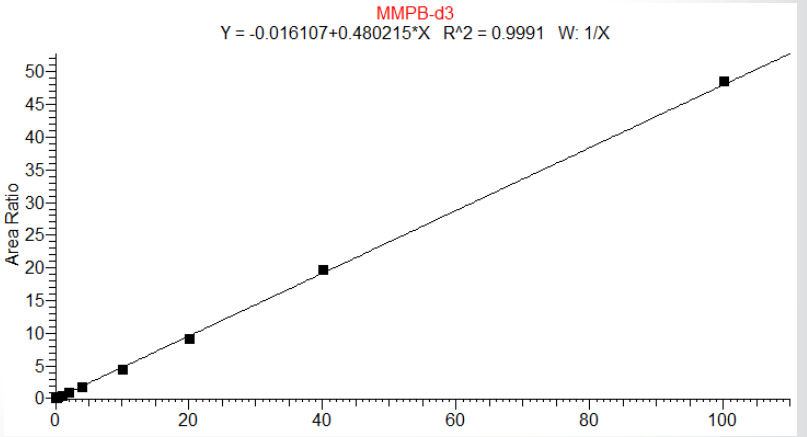
MMPB Method Analytical Details

- Straightforward chromatography (direct injection of 20 uL of sample, C-18 column)
- Analysis in negative ESI mode
- Calibration is linear over at least 4 orders of magnitude
- At present no CRMs are available for MMPB or MMPB-D_3



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.

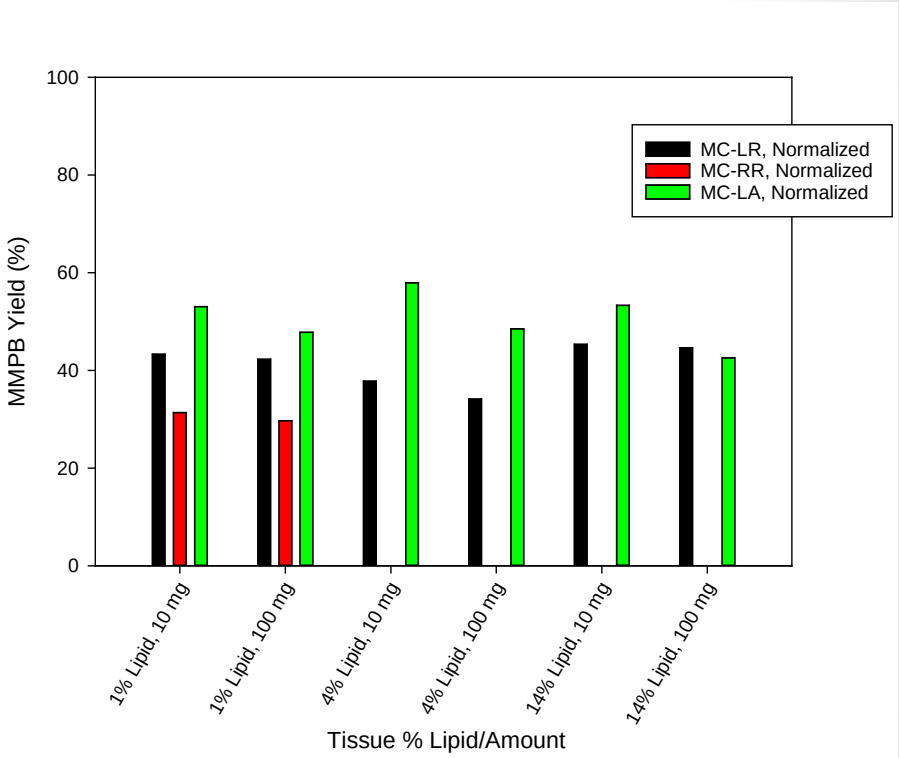
Sample #	MMPB Spike (ng):	MMPB-D3 Spike (ng)	Fish (mg)	Lipid %	MMPB % Recovery
1	40	40	10	4	85
2	40	40	100	4	102
3	40	40	10	14	84
4	40	40	100	14	73
5	4	4	10	4	81
6	4	4	100	4	61
7	4	4	10	14	87
8	4	4	100	14	79
9	40	40	0	na	102
10	4	4	0	na	83

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 yield were not significant
  - Reaction workup did become more laborious with higher fat content, but surrogate recoveries accounted for this.



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

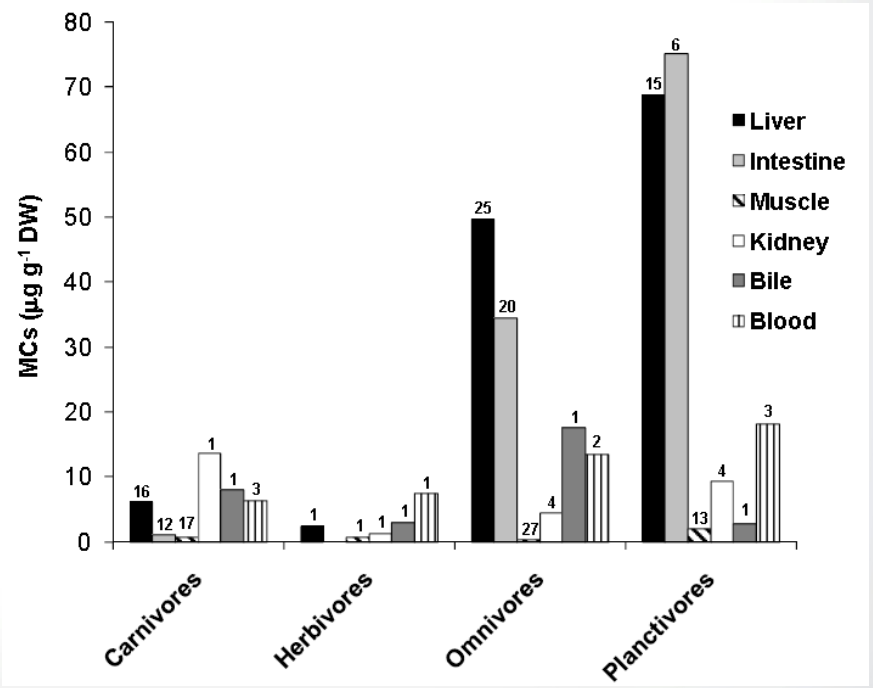
- Mixtures of microcystins were also tested to see if hydrophobicity/hydrophilicity would influence yield
- MMPB yields for MC-LA and MC-RR were not significantly different from 1 to 14% lipid content in the spiked tissue.
- Overall yields were typically 30-40% MMPB based on spike amounts
- Some discrepancies 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

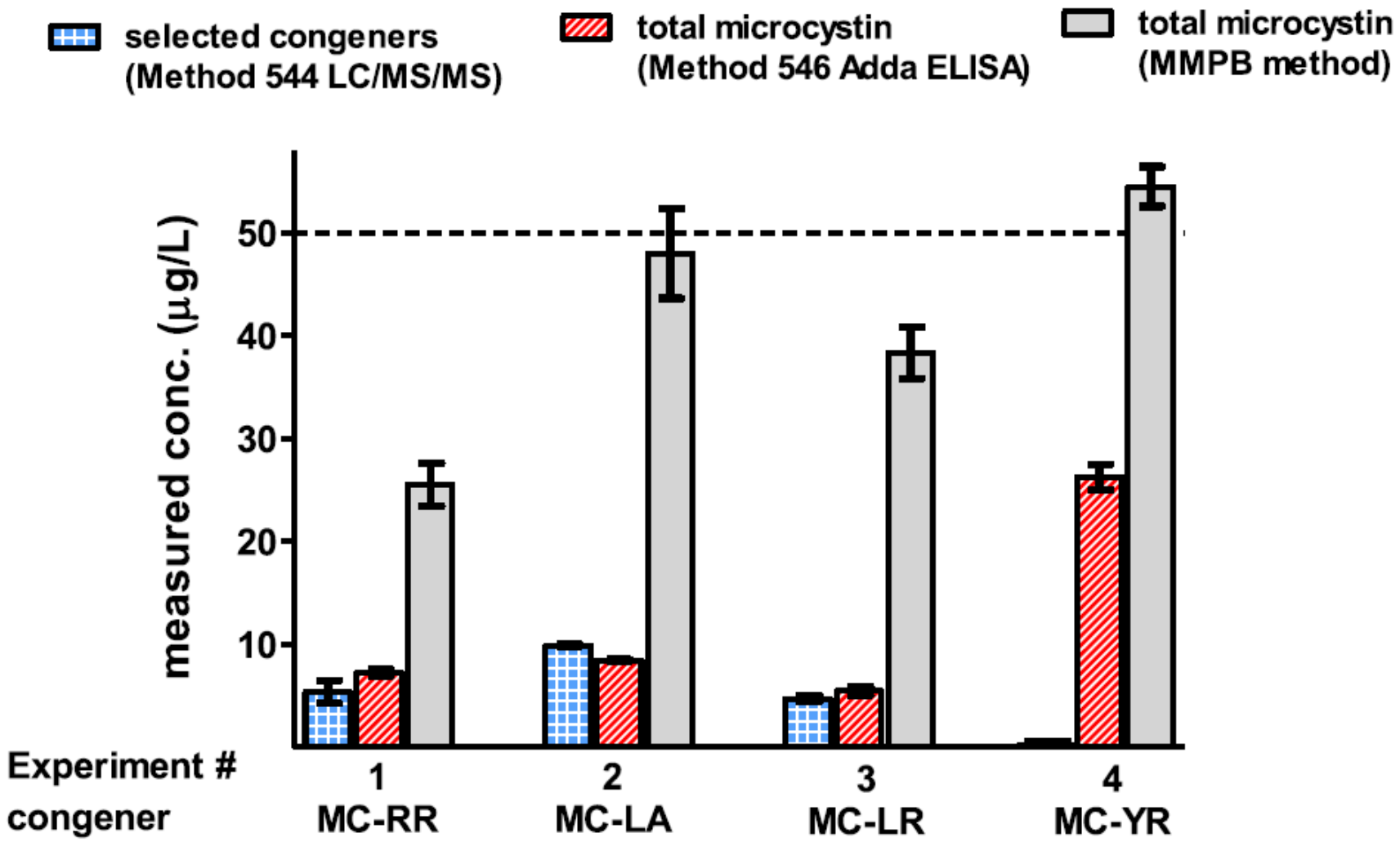
- Working on applying the method to field studies
  - Spiking tissue may not adequately represent state of bioaccumulated toxins, particularly concentration in organs or fats
- To-date have tested carp from a fish kill (negative) and fathead minnows from an on-site study where a bloom was observed (positive)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

Sample:	[MMPB]	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



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

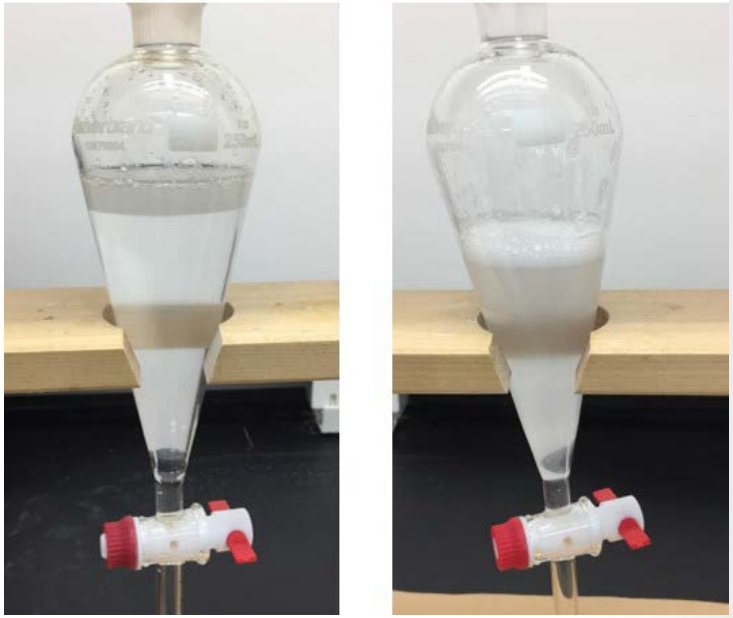
MMPB Method is inappropriate for drinking water studies or chlorinated/oxidized systems



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

Conclusions

- The MMPB technique can be reliably employed for microcystin quantification in fish tissue
- 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
- For tissue quantification the MMPB method provides considerable improvements over extraction of individual toxin congeners and is consistent even with very polar or hydrophobic MCs



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

Acknowledgements & contact information

- Acknowledgements:
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- Contact:

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