Development of methods for measuring total microcystins in Fish Tissue using the 2-methoxy-3-methyl-4-phenylbutyric acid (MMPB) procedure.

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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.

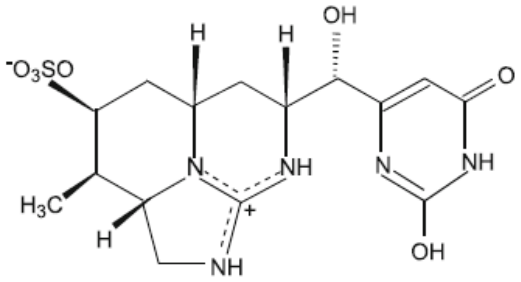
HABs have become a recurrent, increasing and widespread issue globally, with negative impacts that include, but are not limited to, public health and environmental risks from toxin(s) production, light attenuation, diurnal swings in pH and dissolved oxygen, offensive tastes and odors, and impaired visual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

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

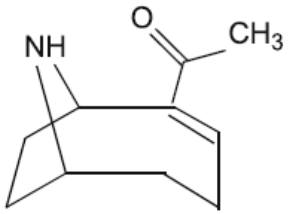
Studies have shown that several algal toxins can cause genotoxic effects, cellular damage and oxidative stress in fish tissues and can accumulate in the muscles, which gives the possibility of human exposure to these toxins through contaminated fish consumption.

Common Cyanotoxins Associated with HABs

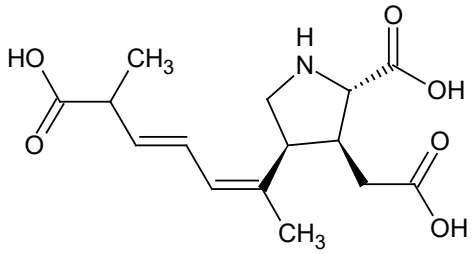
United States Environmental Protection Agency



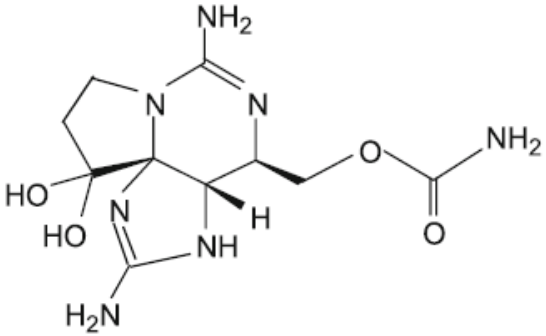
Cylindrospermopsin
Target organs: Kidney, liver



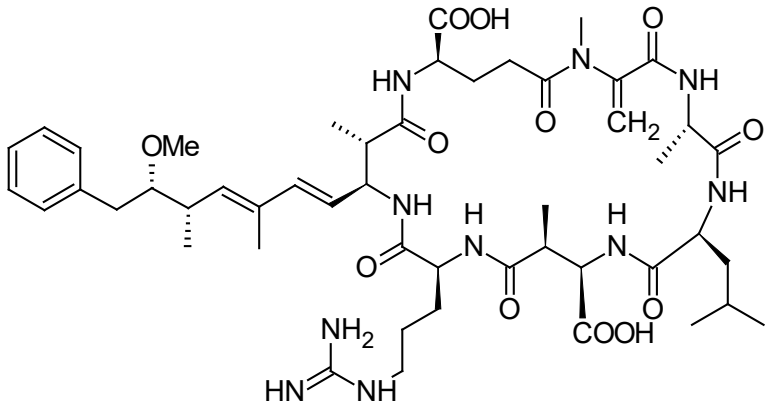
Anatoxin-A
Targets CNS



Domoic Acid
Neurotoxin/Amnesic Shellfish Poisoning



Saxitoxin
(+ Gonyautoxin, other related paralytic shellfish poisons)



Microcystins, more than 200 congeners.
Hepatotoxic, probable carcinogen.
0.3 ug/L health advisory level

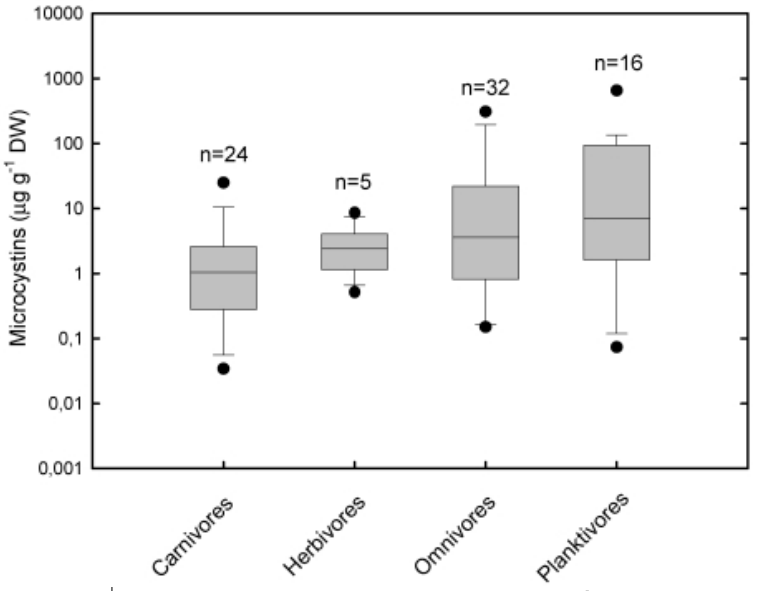
Bioaccumulation/Biomagnification risks of Cyanotoxins

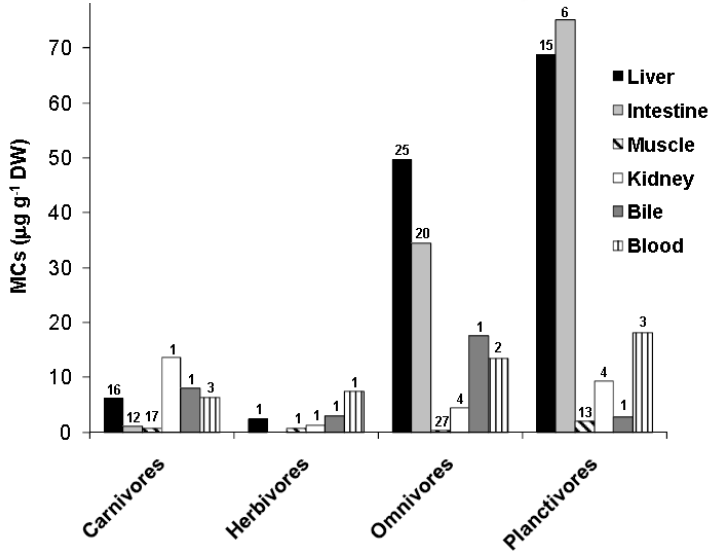
Potential for bioaccumulation or biomagnification of cyanotoxins in food web

- Bioaccumulation from consuming cyanobacteria or toxins in environment
- Biomagnification from persistence in prey species

Shellfish/Clams are known to bioaccumulate saxitoxins and other PSPs

Human health risks from consumption – where do toxins accumulate in tissue? "Are the fish safe to eat" Post-Bloom?





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

Analytical Methods for Cyanotoxins

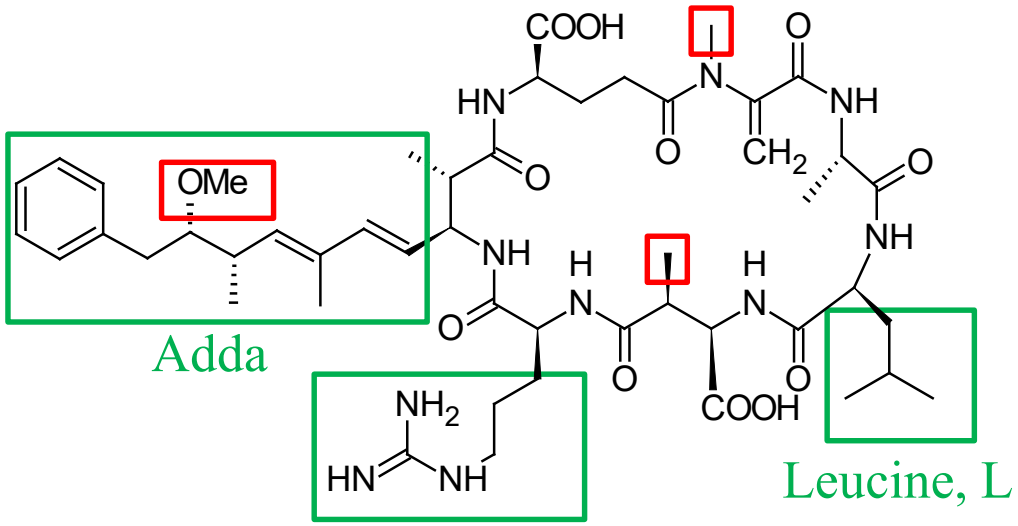
Toxins	Analytical Techniques
• Anatoxin, Cylindrospermopsin	• EPA Method 545 (LC/MS/MS), ELISA
• Domoic Acid	• ELISA, LC/MS/MS
• Saxitoxin	• ELISA (analyte stability issues, no standard method)
• Microcystins	• EPA Method 546 (ELISA), EPA Method 544 (LC/MS/MS), LC/PDA

Microcystins – An analytical challenge

- > 200 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

Microcystins – Analytical Methods

- **ELISA**
  - EPA Method 546
  - Narrow, 0.3 – 5.0 ug/L range of quantitation
  - Cross-reactive across different congeners
  - May be cross-reactive to degradates, chlorinated, other transformed MCs
  - May have significant matrix effects, sensitivity to methanol
- EPA Method 544 (LC/MS/MS)
- Direct inject LC/MS/MS

Microcystins – Analytical Methods

- ELISA
- **EPA Method 544 (LC/MS/MS)**
- Direct inject LC/MS/MS

- Much lower detection limits (low ng/L)
- Targets 6 microcystin congeners and nodularin
- Solid phase extraction method, intended for drinking water
- Application to other matrices challenging or impossible
- In limited use due to logistical challenge of SPE

Microcystins – Analytical Methods

- ELISA
- EPA Method 544 (LC/MS/MS)
- **Direct inject LC/MS/MS**

- Detection ranges vary by instrument and lab, but generally ~0.1 ug/L lower limit
- Susceptible to significant matrix interferences
- Limited by availability of congener standards
- In widespread use, but no standard methods

Microcystins – Analytical Methods

No single, perfect method at present

Challenge of recovering 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.

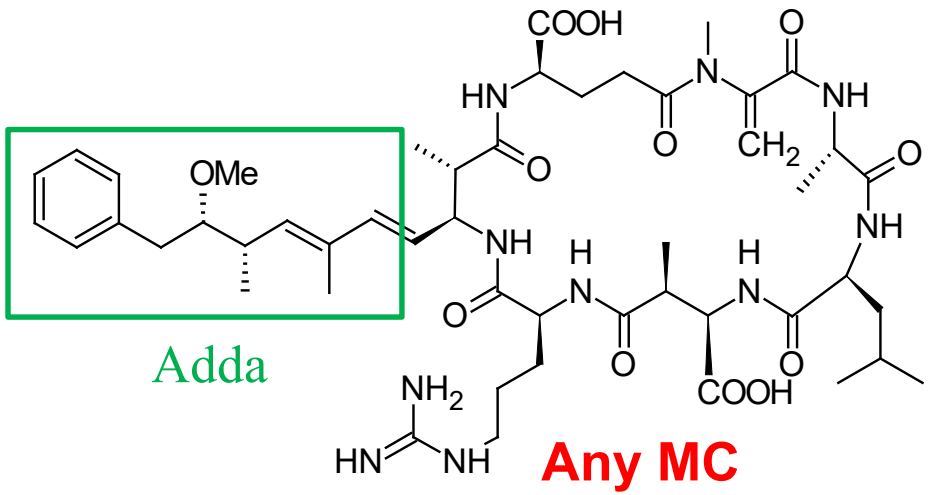
Analyte	Method 1: original QuEChERS (n=5)	Method 2: MeCN (n=5)	Method 3: MeOH (n=15)	Method 3: MeOH with filtration (n=15)	Method 4: MeCN (n=42)
MC-RR	*59*±12	*58*±1	94±*33*	86±*50*	*130*±16
Nod-R	*67*±16	*61*±13	72±18	91±17	94±10
MC-YR	82±9	74±7	*66*±17	94±19	97±17
MC-LR	90±6	*69*±14	*66*±17	89±*34*	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

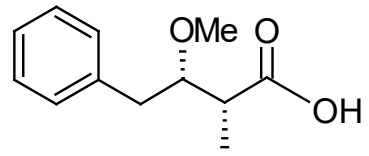


Adda

Any MC congener

$KMnO_4/NaIO_4$

Or Ozone, OsO_4



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

One common product

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

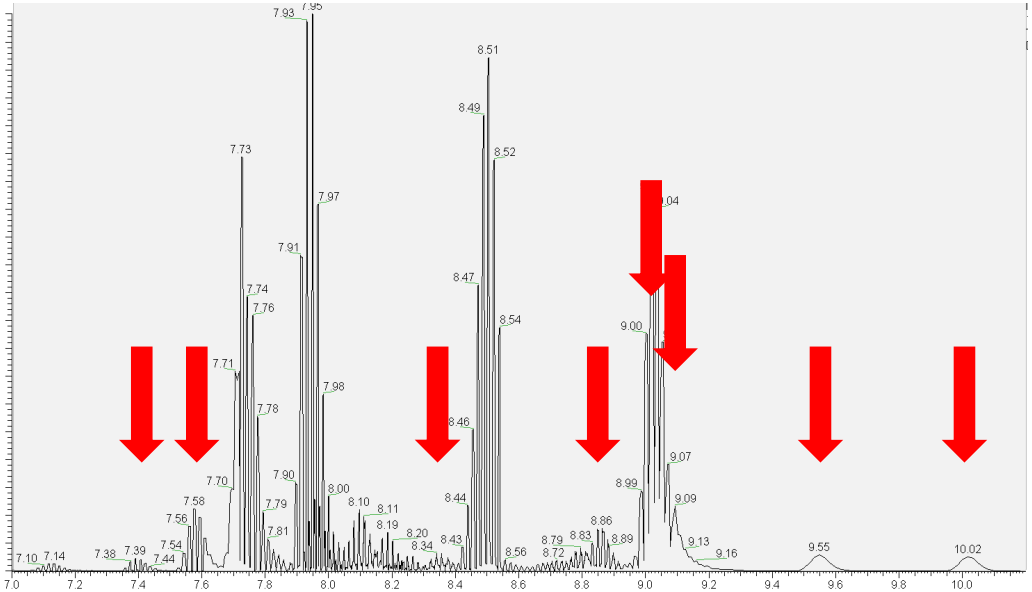
- Simplifies analysis, many congeners to one measurable product
- Cross-reactive with all microcystins containing Adda
- Simplifies extraction from complex matrices (surface water, tissue)

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

EPA United States Environmental Protection Agency

Study Goals

- Optimize MMPB reaction conditions
- 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

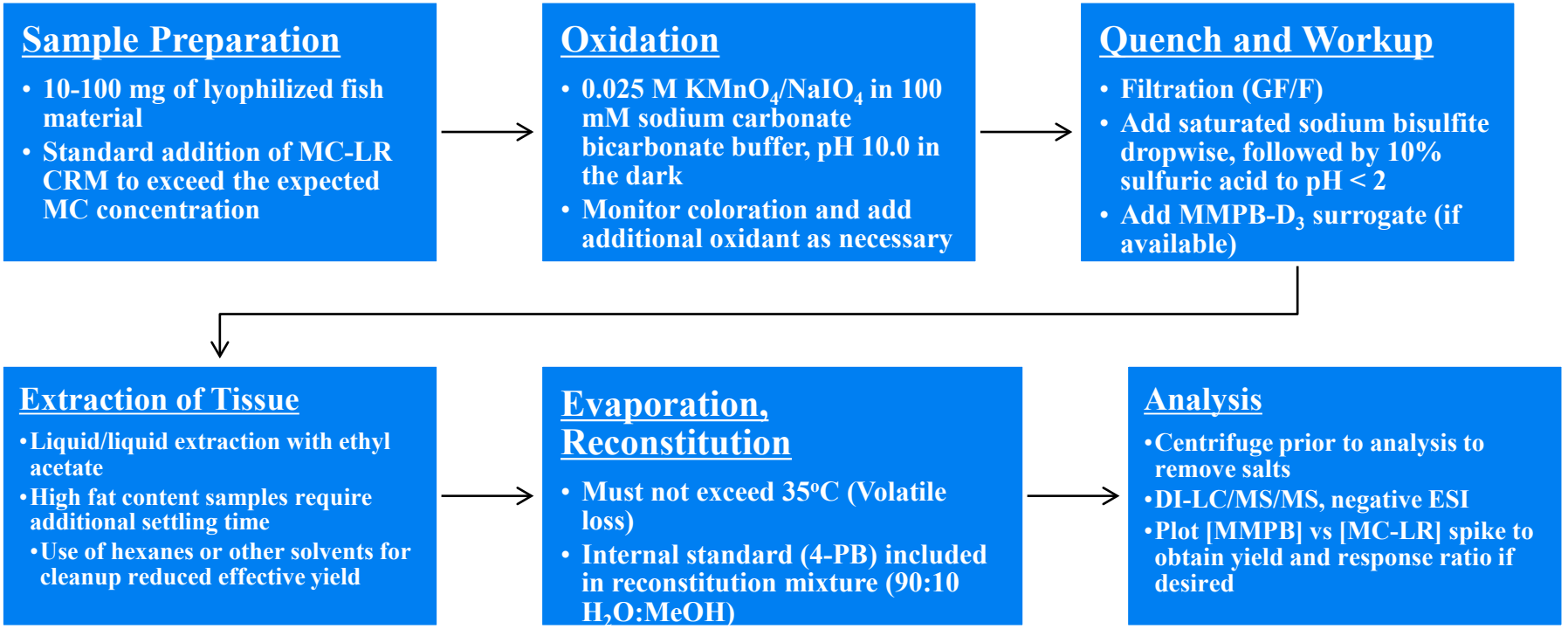
MMPB Oxidation Procedure

- Evaluated oxidant ($KMnO_4$/$NaIO_4$) concentration
- Reaction pH, buffering
- Reaction time
- Temperature

- Workup procedure:
- Quenching
- Extraction, either SPE or liquid-liquid

Optimized MMPB Method Workflow

United States Environmental Protection Agency

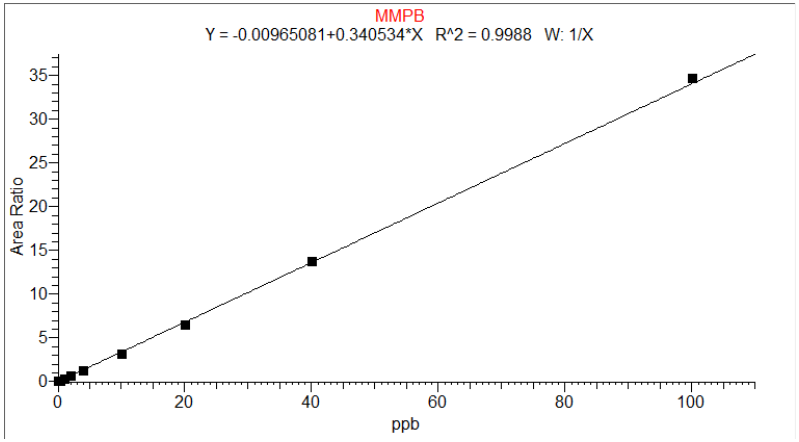


(Recovery of individual MC congeners would use a similar workflow, but omit the oxidation/quenching steps, and efficiency would depend on congener)

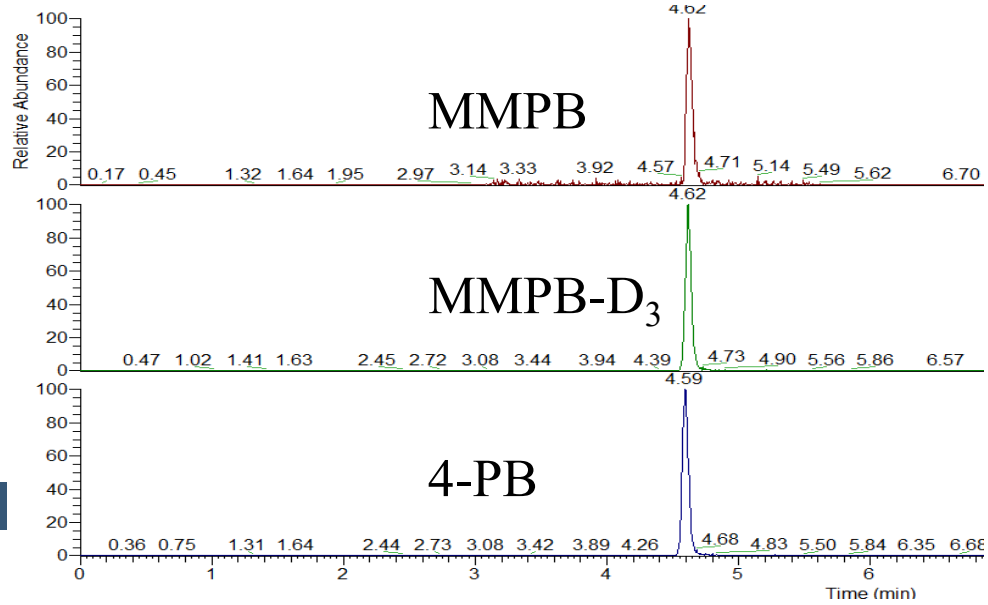
Liquid/liquid conditions similar to Sauve, *et. al. Analytical Chimica Acta, 2014.*

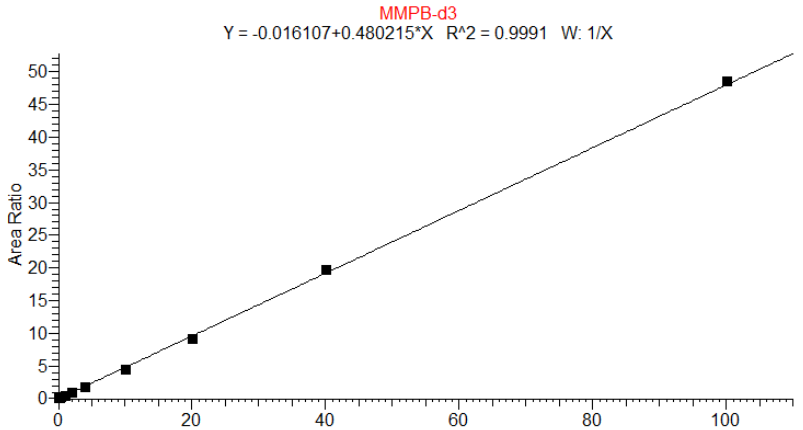
MMPB Method Analytical Details

- Simple water:methanol gradient, analysis by negative electrospray.
- MMPB and MMPB-d_3 used as the analyte and method surrogate, respectively. The latter is added after oxidation, before extraction.
- 4-Phenylbutyric acid used as an internal standard. But fairly high 5-10 ug/L concentration needed for acceptable response.



MMPB Calibration Curve, 0.1 to 100 ug/L





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

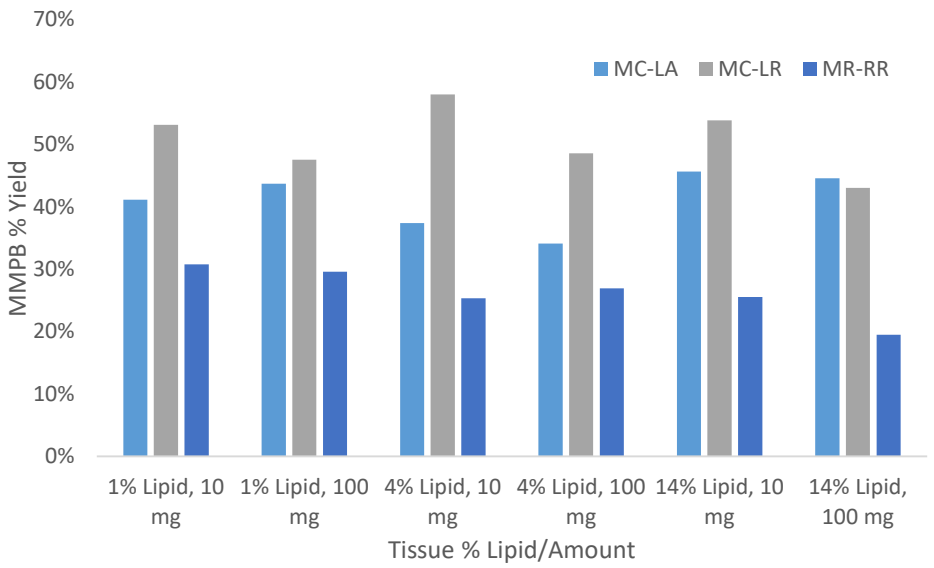
MMPB Application to Fish Tissue – MMPB Spike/Recovery Studies

- Spikes at high (40 ng) and low (4 ng) MMPB and MMPB-D_3 were performed to evaluate extraction performance. Tissue samples were spiked with MMPB, then exposed to oxidation conditions.
- 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 with high effective oxidant concentration

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 with MC-LA, LR, and RR, followed by MMPB procedure
- Three fish matrices were tested: largemouth bass, brown trout, and channel catfish, with 1%, 4%, and 14% lipid content, respectively)
- Significant variance by congener, but this may arise from standard purity (CRMs were not used). Variance by lipid content was smaller



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

- Mixtures of microcystins were also tested.
- 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-RR 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 – 2017 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
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

MMPB Application to Fish Tissue – 2018 Field Studies

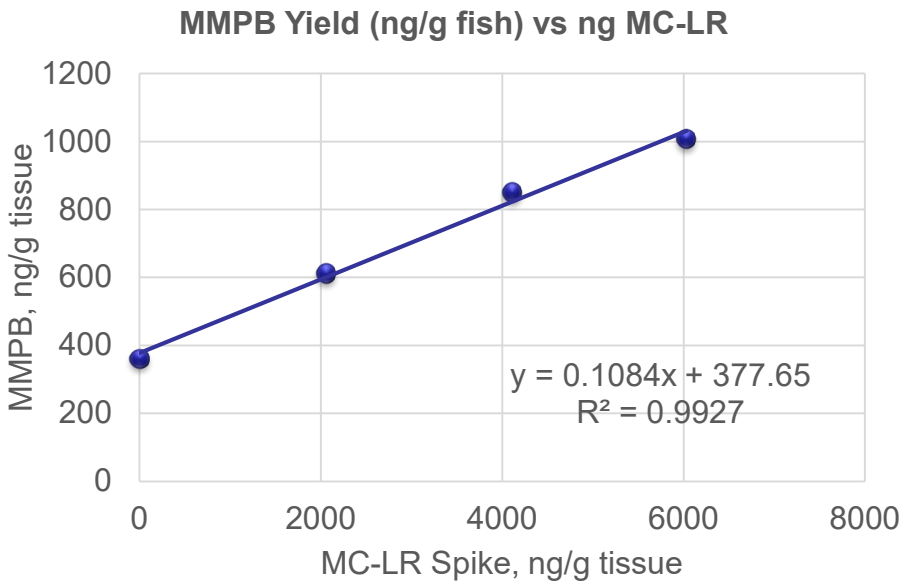
Batch Description	Number of Samples	Status
Periphyton slurry-ESF-8/23/2018	10	Slurry samples frozen (lab-528)
Periphyton slurry-ESF-9/4/2018	10	Slurry samples frozen (528)
Utah Lake-9/5/2018	15	Filleted, homogenized and frozen (528).
Okeechobee, FL-9/12/2018	29	Filleted, homogenized and frozen (528).
Minnows-ESF-9/12/2018	14	14 tissue sample and 7 innards extracted. Instrument was down. Therefore, extracts were blown down to dryness and frozen (528).
Microcystin chronic-10/30/2018	288	Slurry/water samples frozen (427)
Bantam Lake, CT – 10/31/2018	11	Filleting in progress (528)
Trenton, NJ – 11/29/2018	12	Whole fish frozen (427)

No detections in any of the minnow ESF samples

Only 2 detects in slurry samples 2.2 and 6.8 ng/g

Standard Addition of MCs to Improve Quantitation

- No standard method approach for MMPB technique at present
- Limited availability of standards also a challenge for inter-lab comparison (no certified materials)
  - Different labs might report wildly different numbers
- Screening with MMPB can be rapid, but accurate quantitation requires standard addition due to varying oxidation efficiencies
- Can we achieve consistent enough oxidation efficiencies to reduce need for standard addition?



3.5 ug MCs per gram of tissue from the x-intercept.

Standard addition of MCs to a series of sample replicates can improve quantitation accuracy

Optimized method produces 10% mass efficiency consistently

MMPB Oxidation Yield

- Can we use an overall process yield to avoid performing standard addition?
- Theoretical Yield:

~1000 g/mol -> 207 g/mol, for ~1/5 mass conversion

- In practice yield is always ~1/10th.

- Add ELISA vs MMPB 1/10th yield comparison chart.

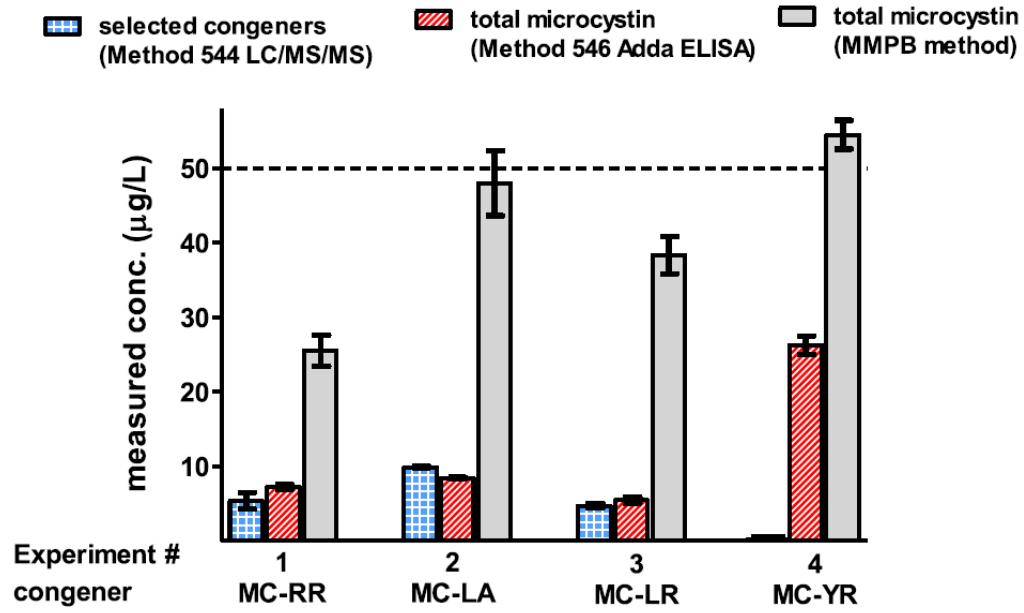
Comparison of MMPB Conversion Yields in Surface Water Matrices

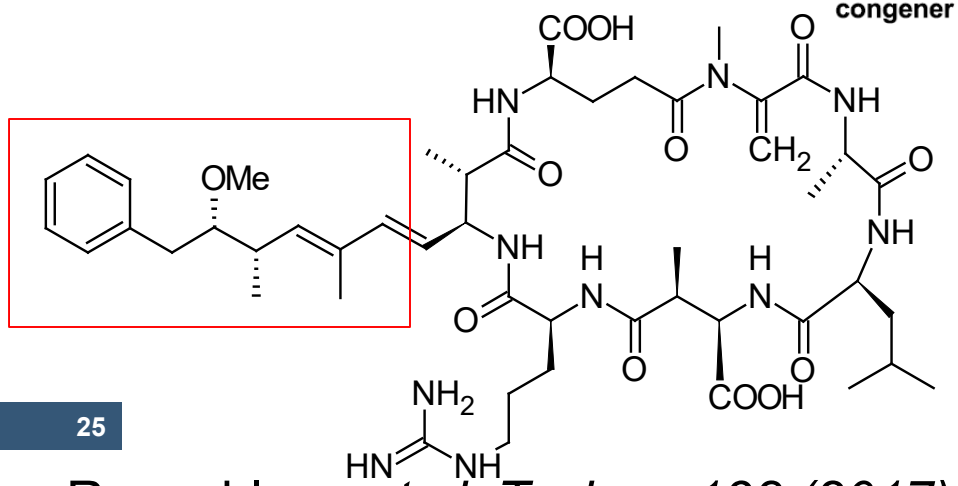
Source	% Yield (Std. Addition)	[MCs] by MMPB, ug/L	[MCs] by ELISA, ug/L	[MCs], Sum of Congeners by LC-MS
Lake 1	65	940	1900	1980
Lake 2	83	2.6	3.2	1.0
Lake 3 (Scum)	75	5120	6200	
Lake 4 (Scum)	67	63000	39000	22300
Lake 5	65	1870	1860	
Lake 6	64	530	490	
Lake 7	50	6.7	14.1	
Lake 7 (Raw)	35	8.1	14.1	1.1
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

Potential Cross-reactivity

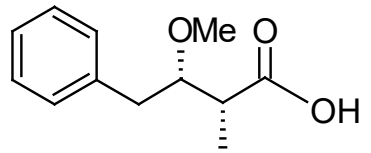
- Cross-reactivity with transformed microcystins
  - Chlorinated water demonstrated
  - Biodegraded species?
  - Metabolites *in vivo*?
- If 'adda' is intact, potential formation of MMPB





$KMnO_4/NaIO_4$

Or Ozone, OsO_4



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

- 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

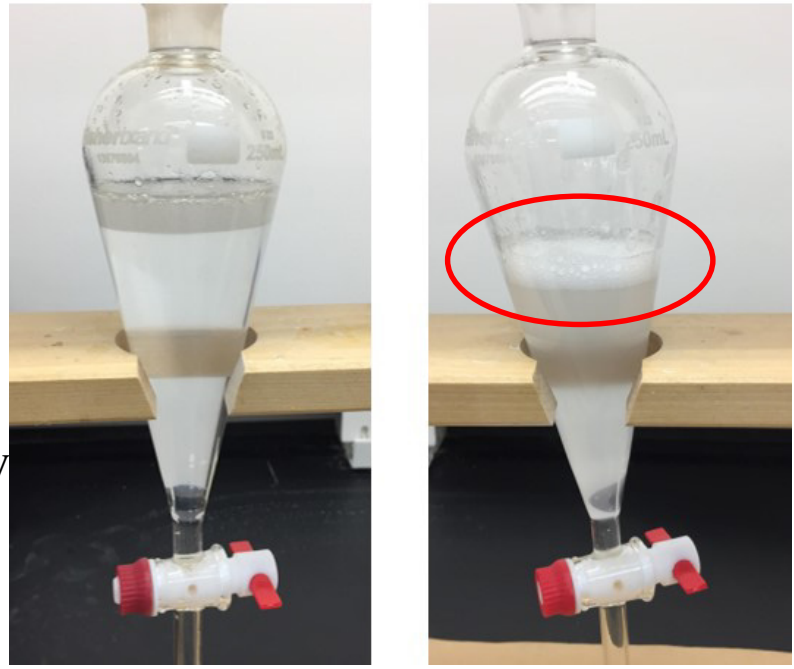
Method quantitation limits of 0.1 to 100 ug/L MMPB correspond to roughly 1 to 1000 ng/g MCs, depending on dilution factors/mass balance

- 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 in fish from high toxin lakes, application to surface and cultures

- 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:
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- Contact:

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