Measurement of "total" microcystins using the MMPB method, and application to HAB impacted surface waters

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Microcystins - Background

- Microcystins are potent hepatotoxins and potential tumor promoters

- Produced by a variety of cyanobacteria associated with inland waters

- The 2014 Toledo “Do Not Drink” advisory resulted from an ELISA-based detection of microcystins in the finished waters of a DWTP

- The ‘Adda’ moiety is the target of the most widely used ELISA method

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Microcystin-LR
Cyclic heptapeptide with many uncommon amino acids

- Adda
- Leucine (L)
- Arginine (R)
Microcystins – An analytical challenge

• **Amino acid substitutions** are most common at two positions but can occur throughout the molecule, and include non-standard amino acids.

• **Desmethylation** is possible at three positions, with *N*-methylation occurring at unsubstituted positions.

• Combined result is over 150 congeners presently identified, many with unique masses and unknown toxicity. Only < 20 are available as standards.

• USEPA 10 day health advisory limit is 0.3 ug/L total microcystins for children, 1.6 ug/L for adults.
## Analytical Methods for Microcystins

<table>
<thead>
<tr>
<th>Technique</th>
<th>Limit of Quantitation</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>ELISA</td>
<td>~0.3 ug/L-5 ug/L</td>
<td>Broadly cross-reactive within MCs</td>
<td>Narrow application range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fast, accessible</td>
<td>May react with non-MCs, precursors</td>
</tr>
<tr>
<td>LC/MS/MS</td>
<td>As low as 0.02 ug/L for 6 congeners</td>
<td>Accurate, precise quantitation</td>
<td>Limited congener availability</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Very sensitive</td>
<td>Can’t prove safety/absence of toxins</td>
</tr>
<tr>
<td>LC/PDA</td>
<td>~0.16 ug/L</td>
<td>May measure unknown congeners</td>
<td>Difficult to quantify, may false-positive</td>
</tr>
<tr>
<td>Protein phosphatase inhibition</td>
<td>0.2 to 2 ug/L</td>
<td>Directly measures one type of toxicity</td>
<td>Narrow calibration range</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reliability concerns</td>
</tr>
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<tr>
<td>Chlorophyll-a, phycocyanin</td>
<td>N/A</td>
<td>Useful for continuous screening</td>
<td>Not specific to (harmful) cyanobacteria, not specific to toxin production</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Inexpensive</td>
<td></td>
</tr>
<tr>
<td>Quantitative (q)-PCR</td>
<td>N/A</td>
<td>Specific to toxin producing cyanobacteria</td>
<td>DNA-based not specific to production of toxins, and may have a long aqueous</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Relatively low cost</td>
<td>lifetime</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>RNA-based time sensitive and not well studied but may be more reactive</td>
</tr>
</tbody>
</table>
How do we prove the safety of a water sample?

- ELISA is frequently employed for routine screening
- Confirmation of detections remains the challenge
  - LC/MS/MS (Method 544) can’t prove absence of congeners outside of the method
  - LC/PDA may not be reliable enough for confirmatory method use
  - PPIA only measure one type of toxicity, and has differential response by congener
  - Molecular methods like PCR are more useful in a surveillance capacity, rather than confirmation at present
  - ELISA is presently being used for confirmation as well
LC/MS/MS screening tools for unknown MCs

- For congeners of identical mass, LC/MS/MS methods can identify presence on chromatography as shoulders or discrete peaks.
- “Parent scan” analysis can identify but not quantify compounds containing “Adda”.
- High resolution, accurate mass instruments might help identify novel congeners, but not quantitatively.
- None of these can provide robust and reliable quantitation of microcystins.
Chemistry of the MMPB Technique

• Application of the Lemieux Oxidation to convert the “Adda” moiety in microcystins to MMPB
• Cross-reactive with all microcystins containing Adda (but may omit congeners with desmethylated Adda), so can potentially measure ‘total’ microcystins present
• Originally developed by Harada in 1996, more recently applied by Foss, et al., to drinking water

Foss, et. al. “Using the MMPB technique to confirm microcystin concentrations in water measured by ELISA and HPLC (UV, MS, MS/MS)”, Toxicon, 2015.
MMPB Method Analytical Details

- Required chemicals include MMPB, MMPB-D$_3$ (surrogate), and 4-phenylbutyric acid (4-PB, internal standard), along with a CRM microcystin standard
  - MMPB and MMPB-D$_3$ standards available from Wako Chemicals (Japan)
  - 4-PB used as an internal standard at 10 ug/L, MMPB-D$_3$ typically used at 10 ug/L as a surrogate prior to workup to verify method performance

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Parent M/Z</th>
<th>Product Ion</th>
<th>Collision Energy</th>
<th>Polarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMPB</td>
<td>207</td>
<td>131</td>
<td>15</td>
<td>ESI-</td>
</tr>
<tr>
<td>MMPB-D$_3$</td>
<td>210</td>
<td>131</td>
<td>15</td>
<td>ESI-</td>
</tr>
<tr>
<td>4-PB</td>
<td>163</td>
<td>91</td>
<td>15</td>
<td>ESI-</td>
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MMPB Method Analytical Details

- Straightforward chromatography (direct injection of 20 \( \mu \)L of sample, C-18 column), water:acetonitrile gradient
- 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\textsubscript{3}

MMPB Calibration Curve, 0.1 to 100 \( \mu \)g/L

MMPB-D\textsubscript{3} Calibration Curve, 0.1 to 100 \( \mu \)g/L

MMPB

MMPB-D\textsubscript{3}

4-PB
Sample Assessment
- Estimate of toxins by ELISA or LC/MS/MS to establish target concentrations for matrix spikes

Preparation
- 1-10 mL of freeze/thawed sample material
- Standard addition of MC-LR CRM to exceed the expected MC concentration

Oxidation
- Initial conditions of 0.015 M KMnO₄/NaIO₄ in 100 mM sodium bicarbonate, pH 8.5 in the dark
- Monitor coloration and add additional oxidant/buffer mix as necessary

Quench and Workup
- Add saturated sodium bisulfite dropwise in a fume hood, followed by 10% sulfuric acid to reduce the pH to ~2, mixing thoroughly
- Add MMPB-D₃ surrogate if available

Extraction
- Liquid/liquid and solid phase extractions were both effective. Which is advantageous depends on the personnel, equipment, and experience of the analyst.

Blowdown, Reconstitution
- Must not exceed 35°C
- Internal standard (4-PB) included in reconstitution mixture (90:10 H₂O:MeOH)

Analysis
- Centrifuge prior to analysis to remove salts
- Plot MMPB response vs MC-LR spikes, calculate ambient MC concentration

SPE Conditions in Foss, et. al., Toxicon 2015., Carmichael et al., Toxicon 2006.

Liquid/liquid conditions in Sauve, et. al.
• Oxidation using a 1:1 mixture of KMnO₄ and NaIO₄ in 100 mM sodium bicarbonate buffer

• Oxidant concentrations varied from 0.015 to 0.1 M KMnO₄/NaIO₄ depending on the oxidant demand
  • Samples with large biomass required additional oxidant addition
  • EPA Method 544 preservatives (particularly trizma) also have a large oxidant demand, complicating split analysis without dilution
Reaction conditions varied from pH 8.5 to 10 in buffered water (100 mM sodium bicarbonate, adjusted with sodium carbonate).

Maximum yield observed at pH 8.5.

Reaction time had little impact on yield, 30 minutes chosen to minimize analysis time.
Following oxidation of a sample, the remaining oxidant must be removed prior to analysis
  - Sodium bisulfite, a reducing agent, is used to quench the oxidant residual

To force MMPB into the organic layer for extraction, the pH is adjusted to ~2
  - SO₂ (gas) is produced, so work was done in a fume hood

Once workup completed, extraction was either liquid/liquid or SPE
  - L/L/E used 3 x Ethyl Acetate
  - SPE used Sep-pak C-18 cartridges
• Spike/recovery studies were performed in reagent water and reaction matrices.

• Recovery of MMPB was comparable between L/L/E and SPE, with and without the presence of reaction materials/byproducts.

• Care must be taken during sample evaporation and reconstitution - Temperatures above 35°C quickly caused loss of MMPB and MMPB-D₃.
Practical Application of MMPB

- Generation of MMPB from MCs is not quantitative
  - Yields of 20-40% are typical
  - Conversion efficiency varies by matrix

- Calculation of MC concentrations requires the use of standard addition of MCs (particularly CRMs) to calibrate the method

- Standard addition implicitly corrects for yield and workup in the method
  - Assumes uniform formation of MMPB by all congeners present

Standard addition curve following addition of 0.5, 1, 2.5, and 5 ug/L MC-LR to a surface water matrix sample negative in MCs

\[ y = 0.9648x - 0.1 \\ R^2 = 0.9561 \]
• Differences in MMPB production might arise from changes in MC solubility or sorption

• MC-LR and MC-LA show comparable response curves for MMPB conversion over a large concentration range

• % Yields are comparable in the surface water matrix studied
Application of MMPB to surface water samples

- Challenges for analyzing surface water samples for toxins include sample preparation of high biomass samples
- Replicate analysis also complex due to heterogeneity of samples
  - One aliquot may vary +/- 100% in sufficiently nasty samples
- MMPB derivatization can potentially simplify the process as it induces lysis and the workup degrades some material
  - More study of high intensity bloom material is necessary to see if results are comparable with separate lysis steps
  - Need high ambient toxin levels for meaningful comparison (curve to right is ~3 ug/L by ELISA, 1.2 ug/L by MMPB, spikes of 20, 40, 80 ug/L
Working Example: MMPB in Surface Waters

- A surface water sample was obtained which contained high MC concentrations by LC/MS/MS and ELISA (1.2-1.5 mg/L, ~7 mg/L, respectively)

- The sample was prepared by 3x freeze/thaw cycles

- Samples prepared at 1/100-dilution in pH 8.5 100 mM sodium bicarbonate buffer (expected MCs are 12-70 ug/L)

- MC-LR spiked at 200, 300, and 400 ug/L in triplicate, with 3 reactions with at ambient concentrations of MCs. KMnO₄ and NaIO₄ added for a 0.025 M final concentration

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<td>LC/MS/MS (Congener-based)</td>
<td>1.2-1.5 mg/L</td>
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Working Example: MMPB in Surface Waters

- Comparing [MMPB] vs [MC-LR] at the X-intercept gives 52.8 ug/L MCs as the diluted MMPB concentration.

- Correcting for dilution gives 5.3 mg/L MCs.

- Actual yield of MMPB is ~40% in matrix, but the standard addition corrects the response.

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Other Practical Considerations

• MMPB can be found in the environment (Foss et al.), and analytical batches should include analysis of unreacted field blanks to prevent false positives.

• In high biomass samples SPE workup may become impossible due to clogging of cartridges.
  • Liquid/liquid extraction can avoid this issue.

• In cases where quantitative accuracy is less important, routine MMPB analysis assuming 20-40% yields could be used for screening.

• Free Adda and/or biodegradates or precursors of MCs could potentially produce positive hits, more study is needed in this area (especially comparison with ELISA, PPIA).

Foss, et. al. “Using the MMPB technique to confirm microcystin concentrations in water measured by ELISA and HPLC (UV, MS, MS/MS)”, Toxicon, 2015.
MMPB for Finished Waters?

- Chlorination can cause rapid degradation of MCs, depending on pH and congeners present
- Intermediate oxidized species may still generate MMPB despite negative ELISA response
- The toxicity of chlorinated MCs may vary by chlorination location and molecular target

\[
\begin{align*}
\text{OMe} & \quad \text{HOCl} & \quad \text{OMe} \quad \text{OH} \\
\text{NaIO}_4 & \quad \text{OMe} \quad \text{HO} & \quad \text{OMe} \quad \text{O} \quad \text{OH} \\
\text{MMPB} & & 
\end{align*}
\]

Tsuji, et al., Toxicon 1997

\[ k' = 0.163 \text{ min}^{-1} \]

\[ k' = 6.05 \text{ min}^{-1} \]

Why use the MMPB Method?

- Probably too complex, hardware and labor intensive for screening, but useful for a better understanding of cyanobacterial toxins (and their precursors/degradates) in the environment
  - Particularly with molecular tools like qPCR could give time-resolved information about toxins in blooms

- If the reaction can be properly validated for precision, labor requirements could be reduced

- Application to drinking waters might be feasible with alterations of reaction conditions
Conclusions

- The MMPB technique can be reliably employed for surface waters with a standard addition procedure
  - Quantitation limits of 0.1 to 100 ug/L MMPB correspond to roughly 1 to 1000 ug/L MCs
  - 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
  - Generally tracks closer to ELISA than LC/MS/MS congener methods, but more study is required

- There is no silver bullet for measurement of microcystins in all target matrices
  - Application of the MMPB method to treated (chlorinated) waters may result in false positives due to intermediate oxidation products
  - USEPA (Office of Water) is presently running a large-scale study on ELISA as an official EPA Method (Method 546?)
Future Work

• Large scale study in progress to compare ELISA, qPCR, MMPB, EPA Method 544 for toxin measurements in Lake Harsha and other sites with high MC concentrations

• Evaluation of alternative oxidation conditions (e.g. Ozone) for practical use on drinking water samples via alternative mechanism

• Ongoing research at USEPA is looking into use of MMPB for recovery of MCs from fish or other tissue samples (Sauve, et al, others)

Sauve et al., J. Ag. Food. Chem. 2015, 63, 7440.
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Thank You!

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