Cross-reactivity of Microcystin Congeners: Effects on Quantification and Monitoring

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OVERVIEW

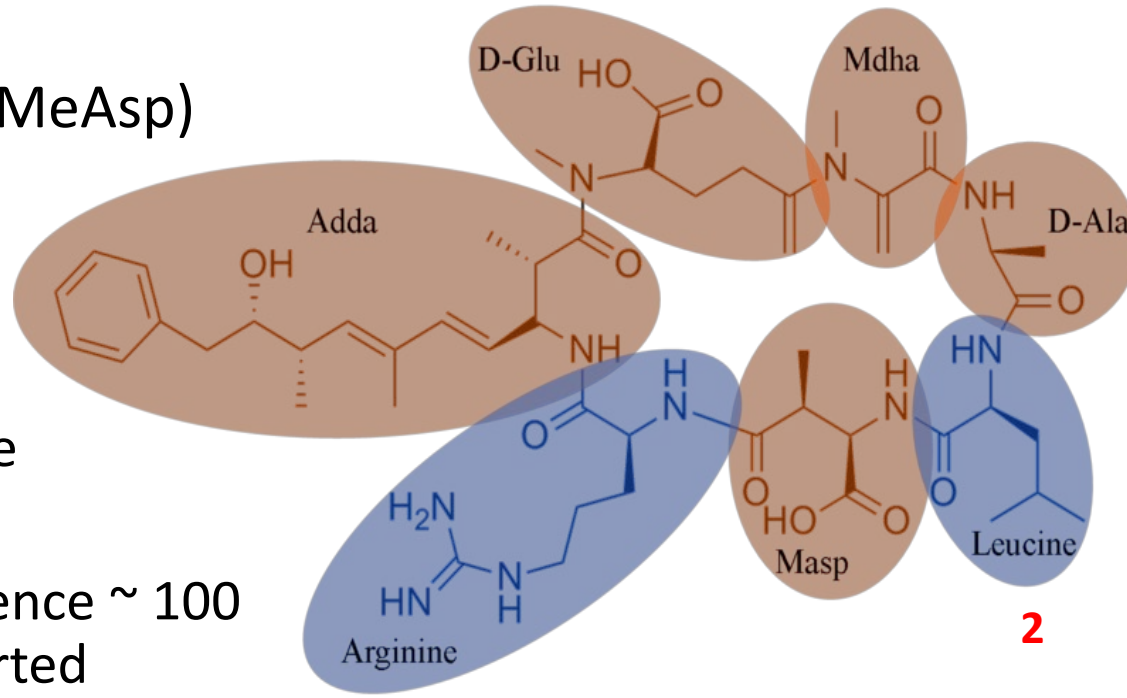
- HABs, Microcystin- Structure, Metabolism and Toxicity
- Methods of Quantification- ELISA
- 4-parametric logistic fit of the MC congeners
- Cross reactivity and EC_{50} of MC congeners
- Interpretation of binding curves and Cross-reactivity
- Toxicity of MC congeners by PPIA

Harmful Algal Blooms

- Cyanobacteria are prokaryotic organisms that cause harmful algal blooms (HAB).
- The eutrophication of lakes, ponds, and oceans favors rapid growth and multiplication of cyanobacteria.
- Complex interaction of several factors such as high concentrations of nutrients, sunlight, temperature, turbidity, pH, conductivity, salinity, carbon availability and slow-flowing/stagnant water can result in the blooms.
- Cyanobacteria produce several secondary metabolites known as cyanotoxins, that are toxic to humans and animals upon ingestion.
- Most commonly observed cyanotoxins are microcystin, cylindrospermopsin, anatoxin, and saxitoxin.

Microcystin

- Microcystins are hepatotoxins.
- It is a cyclic heptapeptide
  - Position 1: D-alanine
  - Position 3: D-erythro-β-methylaspartic acid (MeAsp)
  - Position 5: unique β-amino acid ADDA
  - Position 6: D-glutamic acid (Glu) at
  - Position 7: N-methyl dehydroalanine (MDha)
- Two variable L-amino acids at positions 2 and 4 of the heptapeptide.
- Several substitutions possible at positions 2 and 4, hence ~ 100 different microcystin congeners that have been reported
- The MC-LR, most commonly observed congener is also observed to be the most toxic.



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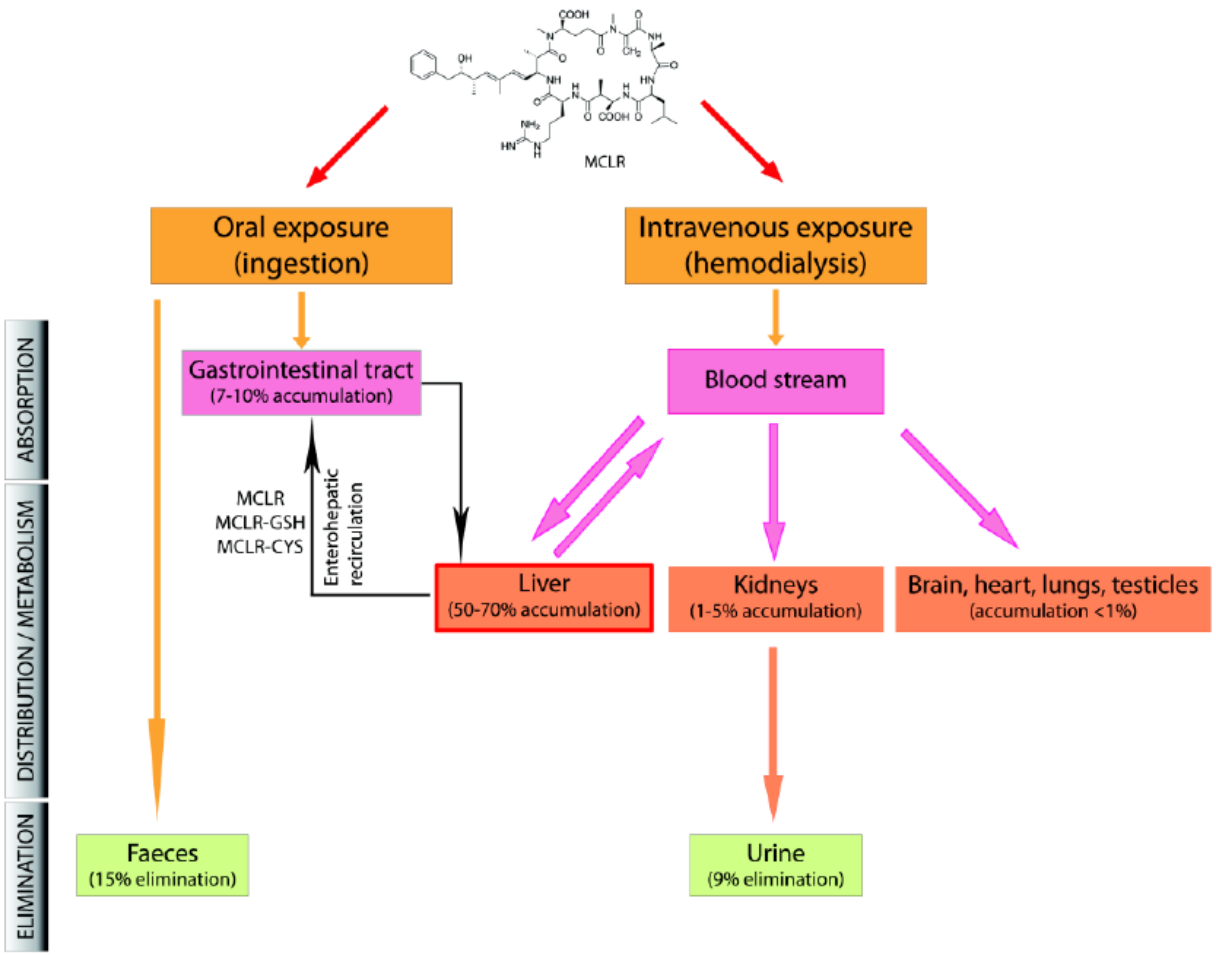
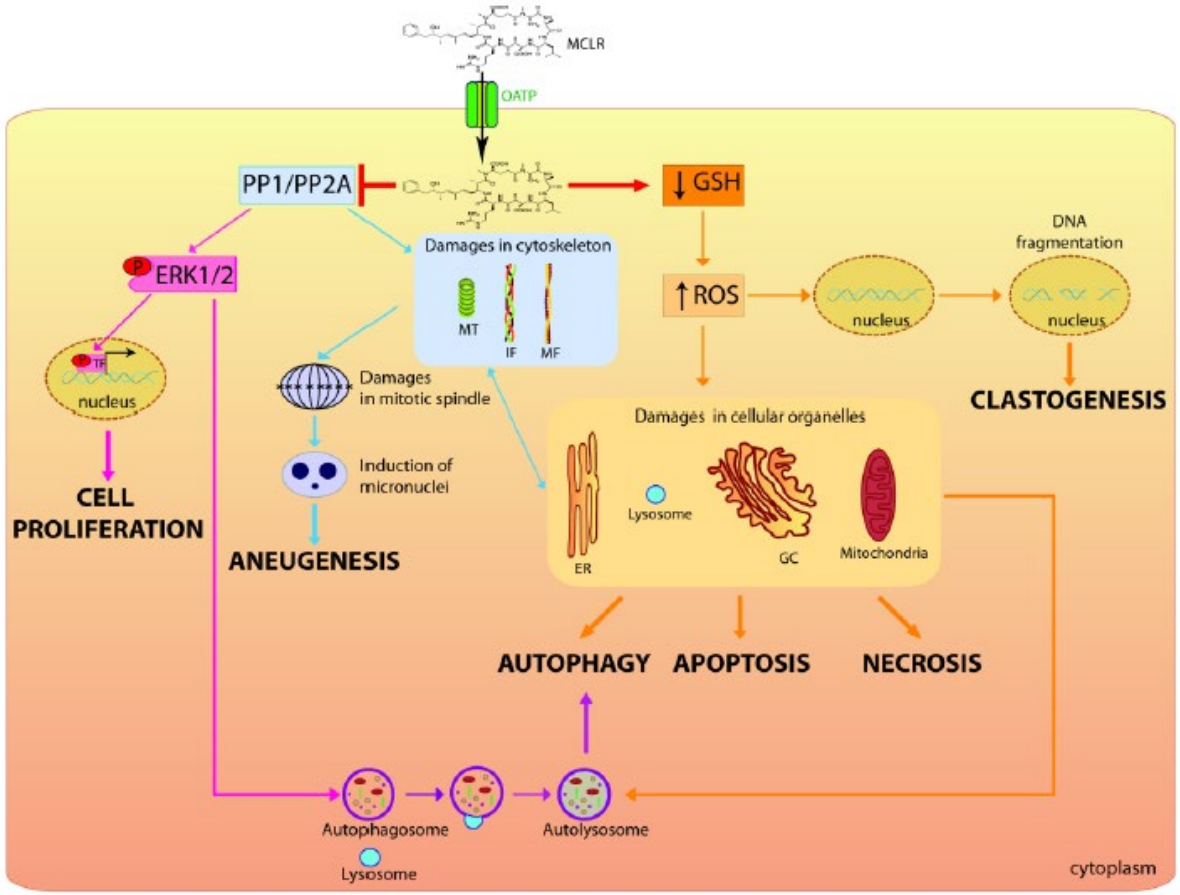
Metabolism and Toxicity of MC-LR



Effects and mechanisms of toxicity of MCLR on Vero-E6 cell model



Carina Menezes, Elisabete Valério and Elsa Dias (2013). The Kidney Vero-E6 Cell Line: A Suitable Model to Study the Toxicity of Microcystins, New Insights into Toxicity and Drug Testing, Dr. Sivakumar Gowder (Ed.), InTech, DOI: 10.5772/54463.

Methods of quantification

- Microcystins can be detected by several analytical methods ranging from
  - **Analytical methods** such as HPLC coupled with UV, PDA or MS detectors, HPLC-MS/MS, MALDI-TOF-MS, GC, CE;
  - **Biochemical methods** such as enzyme-linked immunosorbent assay **(ELISA)** and protein phosphatase inhibition assay **(PPIA)**
  - **Molecular methods** such as quantitative polymerase chain reaction (qPCR).
- Most frequently used methods are ELISA and LC-MS/MS and qPCR methods.
- Each of the methods has their own advantages and disadvantages in terms of cost, time, and detection limits.
- A combination of the methods is often used to quantify Microcystin in surface and drinking water.

Abraxis ADDA-ELISA

- The Abraxis Total Microcystin and Nodularin ADDA-ELISA assay is an **indirect, competitive ELISA**, that uses a polyclonal antibody to target the ADDA moiety.
- MCs present in a sample compete against the MC analog immobilized on microtiter plate for polyclonal anti-MC (and nodularin) antibodies.
- Total MC concentration is then determined by interpolation of a 4-parameter logistic curve prepared with kit-supplied MC-LR standards.
- Total MC results are therefore reported as 'MC-LR equivalents' irrespective of the congeners present.
- **Drawback:** Can detect non-toxic free ADDA, produced by microbial degradation of MCs and the linearized seco-MCs which can be biosynthetic intermediates or hydrolytic by-products

CAAS

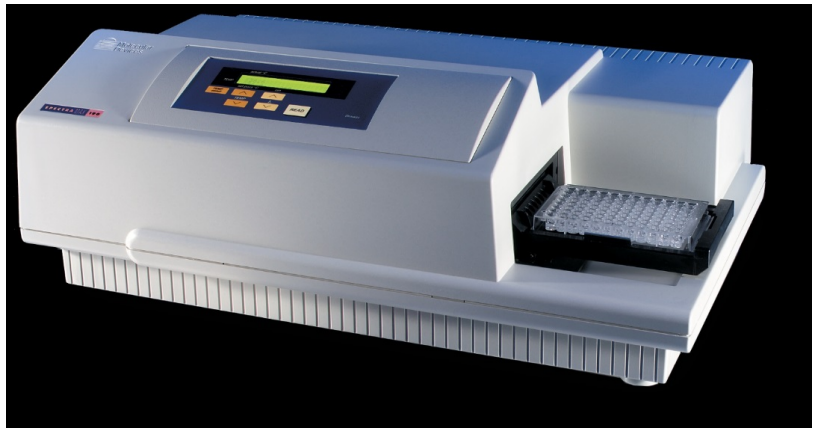


Plate Reader

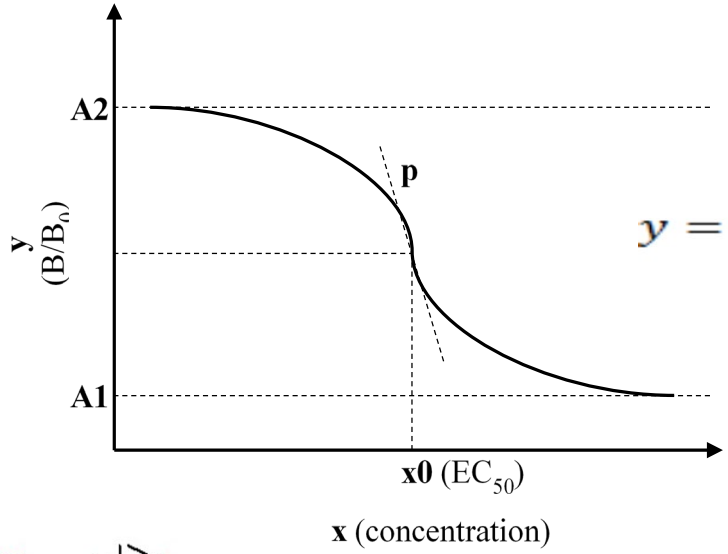
ADDA-ELISA Method Variability

The accuracy of ELISA analysis is highly dependent upon:

- Calibration curve fit and Equivalent Concentrations (EC)
- Storage conditions of the test kit
- Reagent, standard, and ELISA kit lots
- Time and temperature sensitive assay (color development critical step)
- Analyst technique
  - Precision and accuracy of pipetting (reagent volumes)
- Use of alternate vendor standards (non-kit)

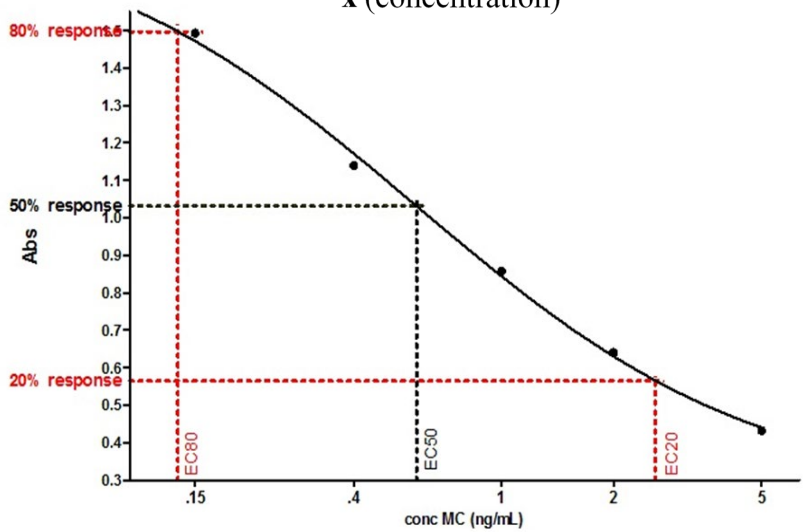
4-parameter logistic fit of the curves

- Calibration Equation
  - y= B/B_0 normalized absorbance;
  - x = concentration,
  - A1 = absorbance at bottom asymptote;
  - A2 = absorbance at top asymptote;
  - x0= concentration at the inflection point (EC_{50});
  - P = slope at inflection point



$$y = \frac{(A1 - A2)}{1 + \left(\frac{x}{x0}\right)^{p}} + A2$$

- Equivalent Concentrations (EC)
  - Concentration on the x-axis related to 20,40,60,80% of the maximum absorbance
  - EC_{20} – Upper limit of useful measurement
  - EC_{40} – Upper limit of most reliable measurement
  - EC_{50} – Concentration at the inflection point
  - EC_{60} - lower limit of most reliable measurement
  - EC_{80} - Upper limit of useful measurement



4-parametric fit vs Log-logit fit

Congener	Coefficient of determination (R^2)	
	4-paramteric	Log-Logit
MC-YR	0.998	0.972
MC-RR	0.990	0.985
MC-LY	0.999	0.975
MC-LA	0.995	0.972
MC-WR	0.999	0.984
MC-LF	0.994	0.988
MC-LW	0.999	0.959
dmMC-LR	0.992	0.946
[D-Asp3]MC-LR	0.999	0.990
[D-Asp3]MC-RR	0.999	0.982
MC-HtyR	0.999	0.973
MC-HilR	0.996	0.991

- The Log-Logit fit is a linear fit derived plotting the Logit vs log of the concentration.
- The Logit function is derived using the equation,

$$Logit = \frac{\log\left(B\,/B_0\right)}{1-\left(B/B_0\right)}$$

- The coefficient of determinations were higher for most congeners using the 4-parametric curve fit compared to the linear fit

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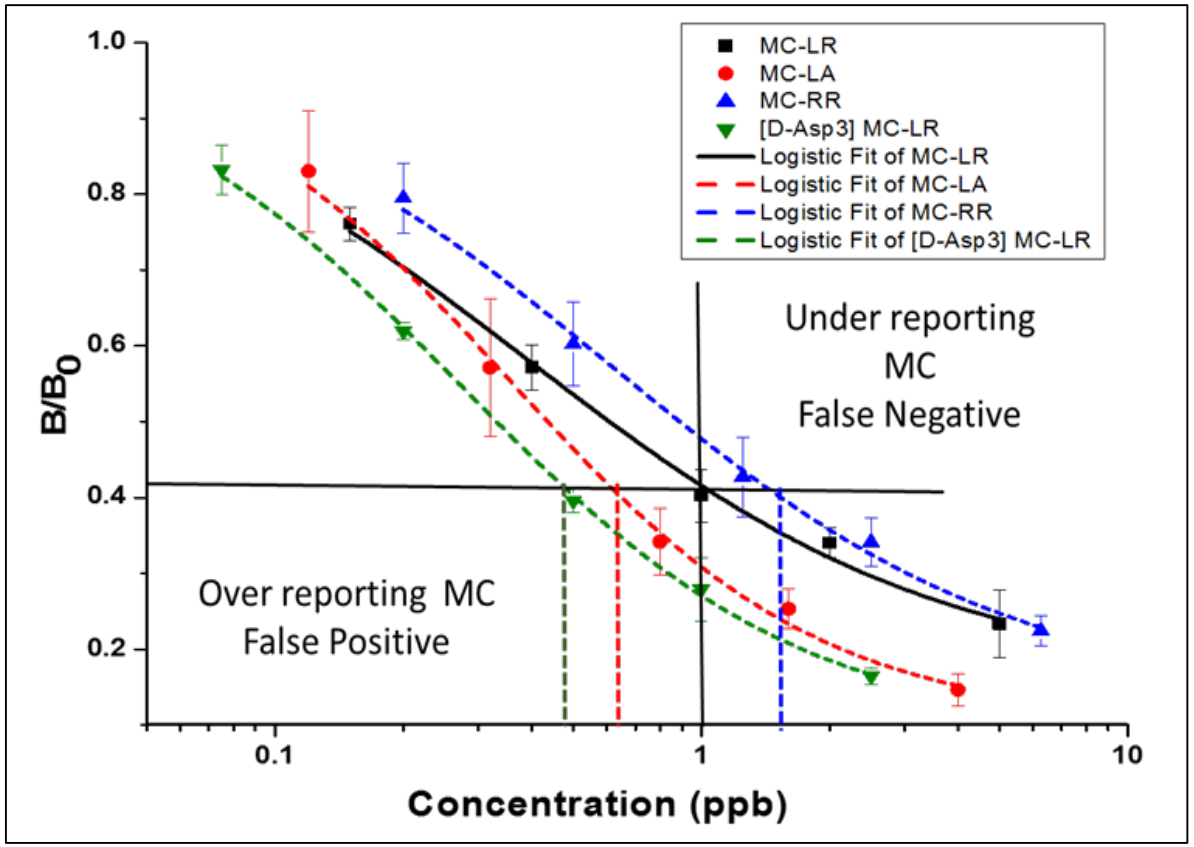
Interpretation of binding curves



- ~ 0.6 µg/L of MC-LA and 0.5 µg/L of [D-Asp3] MC-LR will be determined as 1 µg/L MC-LR equivalent
- MC-RR congener would be underestimated where ~1.5 µg/L MC-RR will be interpreted as 1.0 µg/L.
- The high affinity congeners when present in a sample can lead to false positives. Whereas lower affinity congeners might lead to false negatives

EC_{50} and %CR

Congener	EC_{50} (µg/L)	NEORSD %CR	Published %CR**
MC-LR	0.39±0.07	100±34	NA
MC-LA	0.35±0.04	111±32	NA
MC-LY	0.32±0.01	122±25	NA
MC-RR	0.63±0.10	63±20	50
MC-RR*	0.65±0.10	60±20	50
MC-WR	0.44±0.02	90±19	NA
MC-LF	0.48±0.08	82±27	108
Nodularin	0.46±0.08	85±29	100
MC-LW	0.37±0.00	106±20	118
dmMC-LR	0.32±0.05	123±39	157
[D-Asp3] MC-RR	0.34±0.01	114±24	NA
MC-HTyr	0.30±0.01	132±29	NA
MC-HilR	0.50±0.09	78±27	NA
[D-Asp3] MC-LR	0.27±0.02	143±33	NA
[D-Asp3] MC-LR*	0.37±0.02	105±24	NA
MC-YR	0.41±0.03	95±23	167
MC-YR*	0.53±0.04	75±18	167

**Source- Fischer et al. 2001 : NA- Not available

- The EC_{50} is the effective concentration halfway between the baseline and maximum absorbance at the inflection point of the curve.
- EC_{50} values reflect the binding affinity of the congeners towards the primary antibody in the assay relative to MC-LR.

$$\%\ CR = 100\% \times \frac{EC_{50}\text{ of MC-LR}}{EC_{50}\text{ of the congener}}$$

- EC_{50}-derived cross-reactivity are used as correction factors to adjust ADDA-ELISA test

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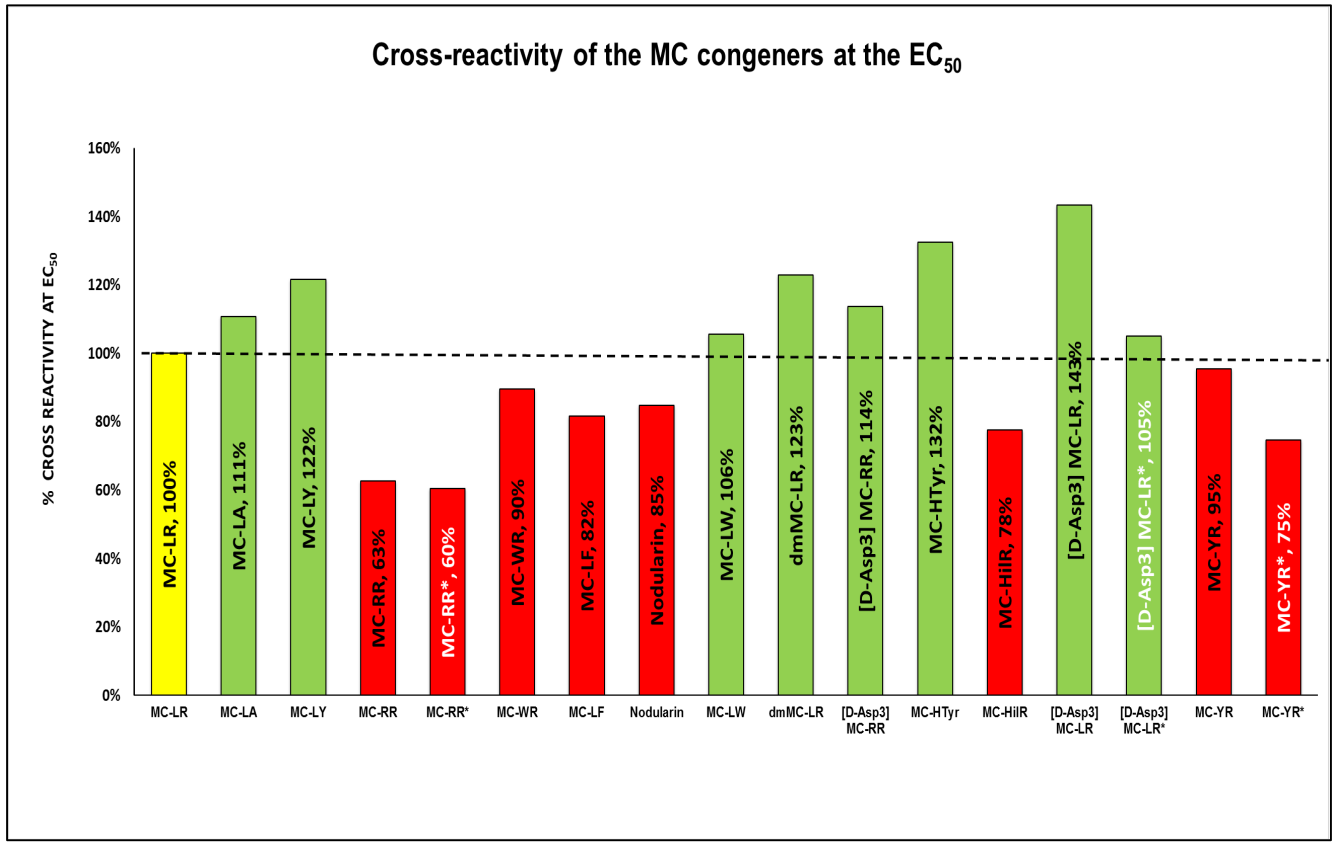
Effect of %CR on MC quantification by ELISA



- 7 congeners exhibited EC_{50} - based % CR > MC-LR standard.
- 6 congeners had % CR's less than that of MC-LR.
- Depending upon the prevailing congener in a sample, results will therefore be under/overestimated.
- A congener with EC_{50} value lower than the MC-LR bind with higher affinity and therefore have higher cross-reactivity.
- The congeners with higher cross-reactivity will be overestimated and lower cross-reactivity underestimated.

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Range of equivalent concentrations

Congener	Equivalent Concentrations (ng/ml)				
	EC_{20}	EC_{40}	EC_{50}	EC_{60}	EC_{80}
MC-LR	1.86	0.62	0.39	0.25	0.08
MC-LA	1.15	0.50	0.35	0.25	0.11
MC-LY	0.96	0.44	0.32	0.23	0.11
MC-RR	2.91	1.01	0.65	0.42	0.14
MC-RR*	2.91	1.01	0.65	0.42	0.14
MC-WR	1.45	0.62	0.44	0.31	0.13
MC-LF	2.10	0.74	0.48	0.31	0.11
Nodularin	2.06	0.72	0.46	0.30	0.10
MC-LW	1.03	0.50	0.37	0.28	0.13
dmMC-LR	1.06	0.45	0.32	0.22	0.10
[D-Asp3] MC-RR	1.02	0.47	0.34	0.25	0.12
MC-HTyr	1.06	0.43	0.30	0.20	0.08
MC-HilR	2.86	0.84	0.50	0.30	0.09
[D-Asp3] MC-LR	0.98	0.4	0.27	0.19	0.08
[D-Asp3] MC-LR*	1.34	0.54	0.37	0.26	0.10
MC-YR	1.33	0.58	0.41	0.29	0.13
MC-YR*	1.70	0.74	0.53	0.37	0.16

- The EC_{20} to EC_{80} is generally considered the optimum range for accurate determination using the 4-parametric fit.
- Beyond this range a ceiling effect is observed in the curves which generally increases the error.
- Interestingly the EC_{20} to EC_{80} range of MC-LR was observed to be from 1.86 to 0.082 µg/L.

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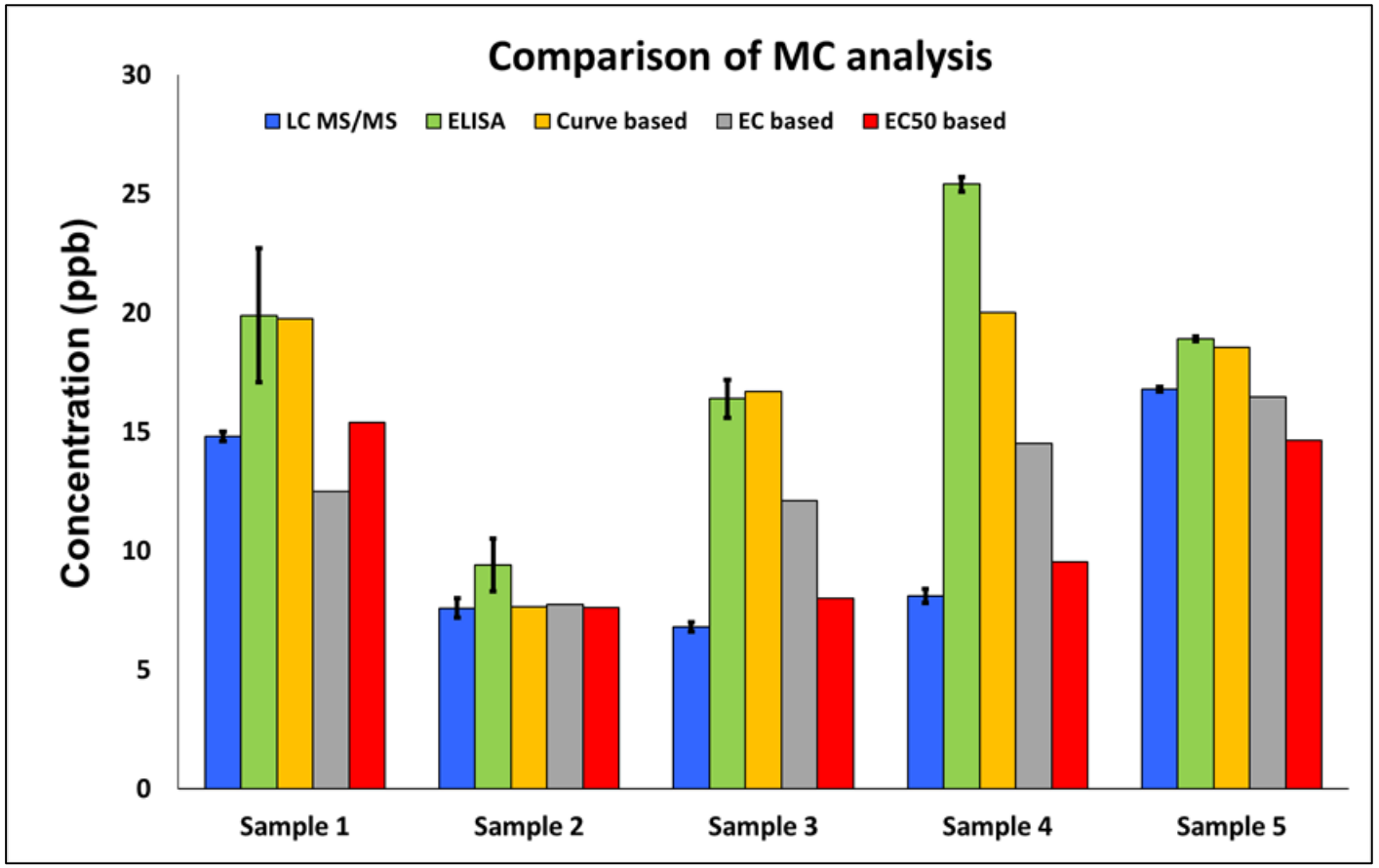
Range of %CR from EC_{20}-EC_{80}

Congeners	% Cross reactivity				
	EC_{20}	EC_{40}	EC_{50}	EC_{60}	EC_{80}
MC-LR	100%	100%	100%	100%	100%
MC-LA	163%	124%	111%	99%	75%
MC-LY	194%	139%	122%	106%	76%
MC-RR	66%	64%	63%	62%	59%
MC-RR*	64%	61%	60%	59%	57%
MC-WR	128%	99%	90%	81%	62%
MC-LF	89%	84%	82%	80%	75%
Nodularin	90%	86%	85%	83%	79%
MC-LW	180%	123%	106%	90%	62%
dmMC-LR	175%	136%	123%	111%	86%
[D-Asp3] MC-RR	182%	130%	114%	99%	71%
MC-HTyr	177%	144%	132%	122%	99%
MC-HilR	65%	74%	78%	82%	93%
[D-Asp3] MC-LR	190%	156%	143%	132%	108%
[D-Asp3] MC-LR*	139%	114%	105%	97%	79%
MC-YR	140%	107%	95%	85%	65%
MC-YR*	110%	84%	75%	67%	51%

- The % CRs were also calculated for the entire EC_{20} to EC_{80} range to further discern differences between congeners and congener concentration.
- % CR varied and tended to be higher towards the extremities (EC_{20} and EC_{80}.) relative to the MC-LR EC_{50}.
- These discrepancies bring into the question the practice of using only EC_{50}-derived cross-reactivity factors for total MC quantification.
- The change in cross-reactivity over the congener concentrations may provide a possible explanation for discrepancies observed due to dilution

Accuracy of correction

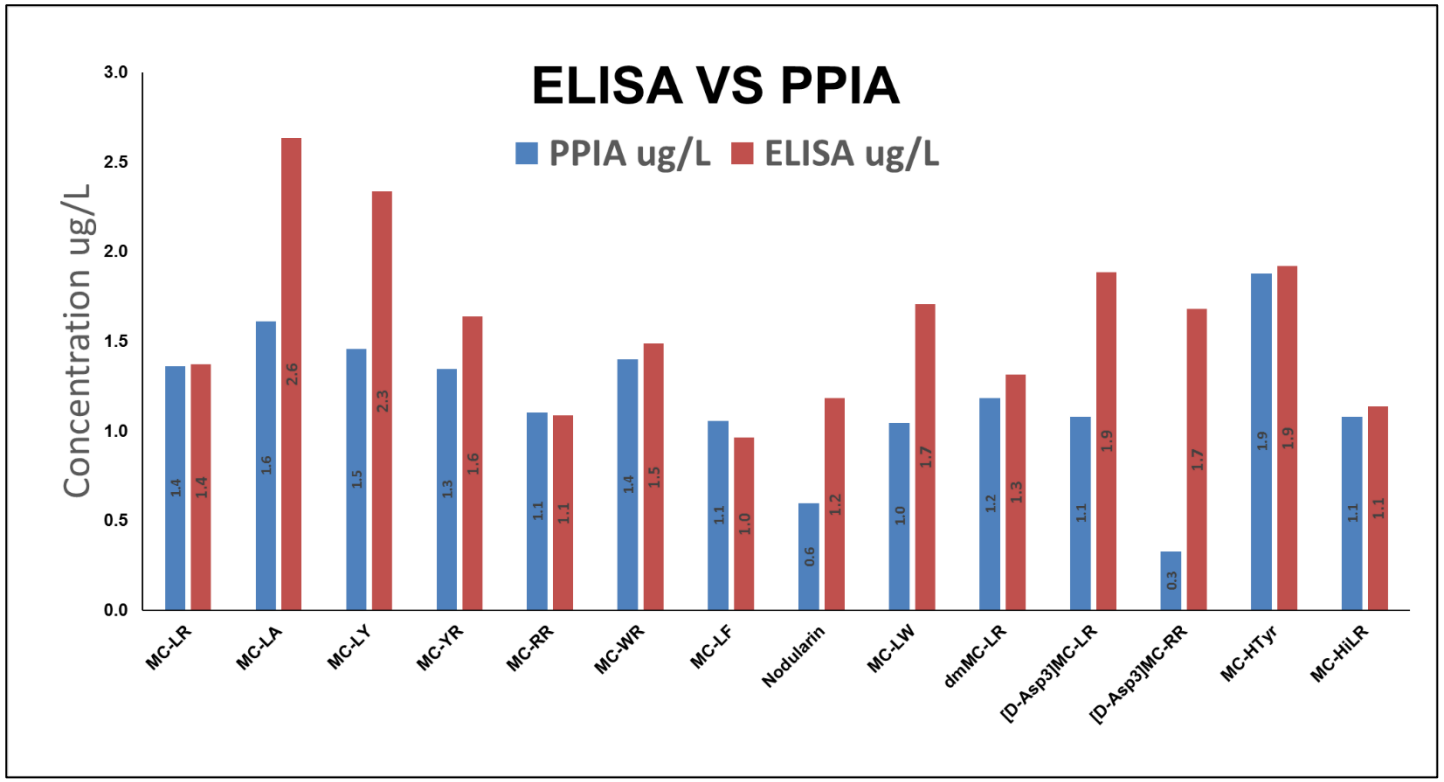


- Samples quantified using the LC-MS/MS and ADDA-ELISA.
- The LC-MS/MS data were corrected in different ways.
  - widely used single cross-reactivity derived from EC_{50} (EC_{50} based).
  - cross-reactivity derived from the nearest equivalent concentration was used (EC based).
  - the equations derived from the binding curves (Curve based).

Protein phosphatase inhibition assay (PPIA)

- The MC and Nodularins are known to be protein phosphatase inhibitors. This property is analyzed by the Microcystins/Nodularins PP2A Kit, Abraxis, Inc. (PN: 520032).
- The phosphatase in the kit hydrolyses a specific substrate that can be detected at 405 nm.
- Samples containing MC will inhibit the enzyme activity proportionally to the amount of toxin contained in the sample.
- Other toxic substances might interfere with the assay and can result in false postives

ELISA vs PPIA of MC congeners



- The toxicity and quantification of MCs by ELISA are two different methods that can have varied results depending on the congener present
- The ELISA quantifies the MCs depending on the structure but is affected by cross-reactivity
- Alternatively, the PPIA measures the cyanotoxins by their ability to inhibit protein phosphatase

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Conclusion

- Differential cross-reactivity exist between the 13 MC congeners studied.
- ELISA assay **MIGHT** over or underestimate the amount of MC present in the sample resulting in both false positives and false negatives.
- Moreover, % CR varied according to congener concentration indicating that the use of a single cross-reactivity correction factor (EC_{50}) may not yield the most accurate results.
- The disagreement in LC/MS/MS and ELISA data can be due to cross-reactivity predominant congeners
- The variation in total MC values with dilution effect can be due to cross-reactivity of the congeners present
- Toxicity results and quantification can vary depending on the congener present

Implications of the study

- The public health implications of these findings have yet to be determined, but could potentially lead to inadequate or inconsequential regulatory and utility response (false negative, risk underestimation) and be detrimental to consumer confidence.

- False positives and overestimates could also be financially burdensome for utilities (unnecessary public notification, implementation of advanced treatment).

Acknowledgements

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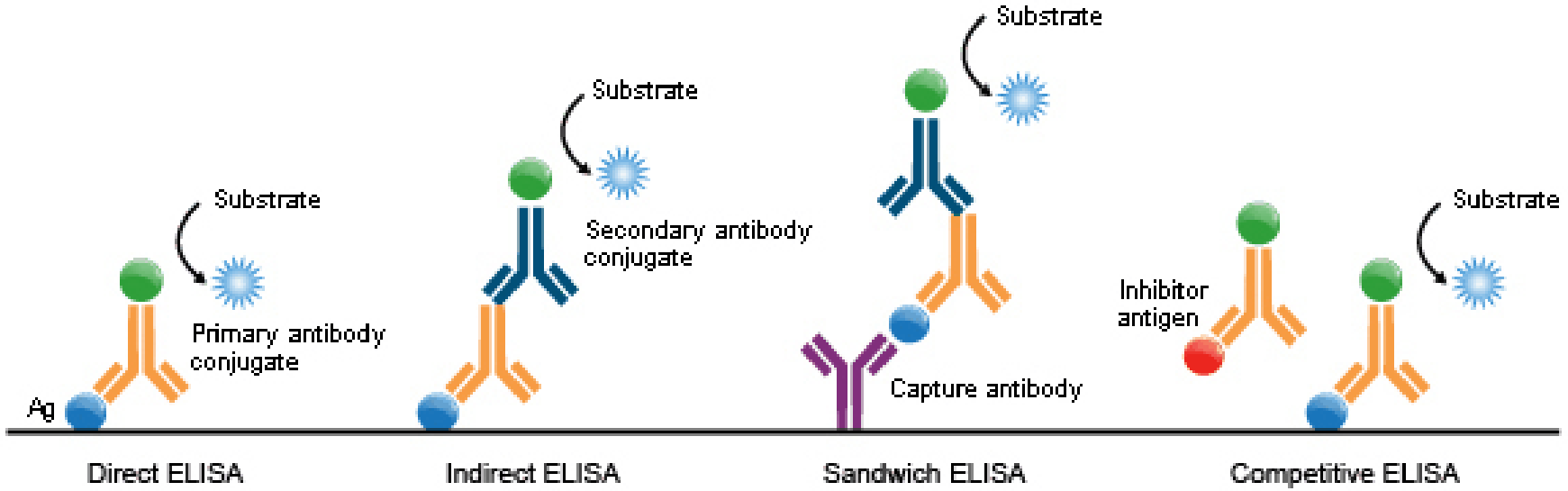
General ELISA Assay Workflow

Types of ELISA



Indirect Competitive ELISA