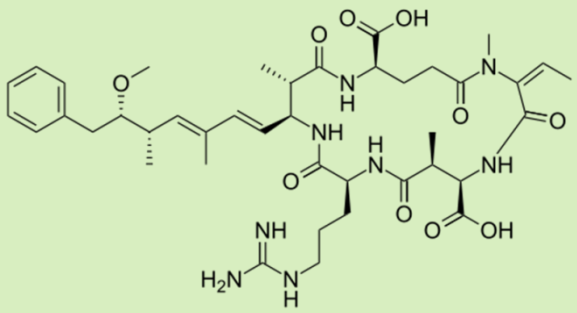
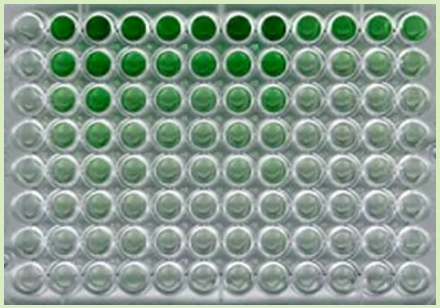
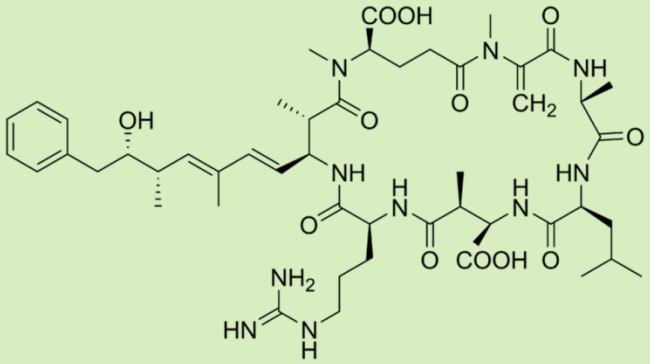
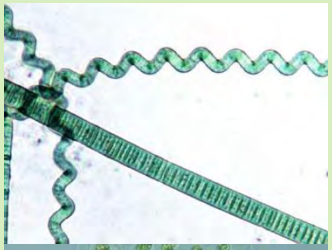
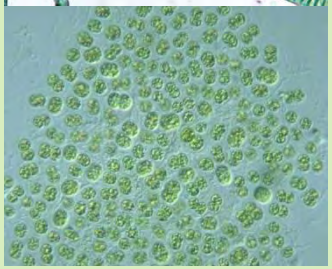
Laboratory Analysis of Cyanotoxins

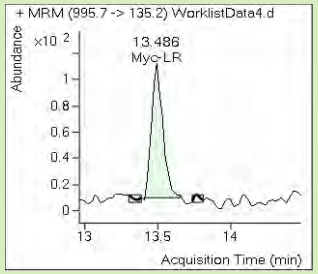


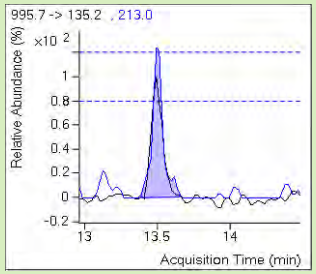


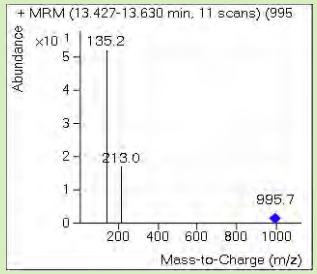


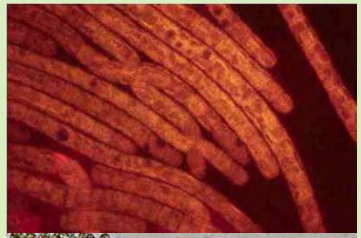


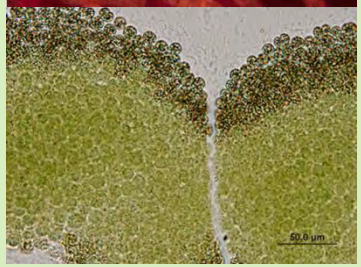




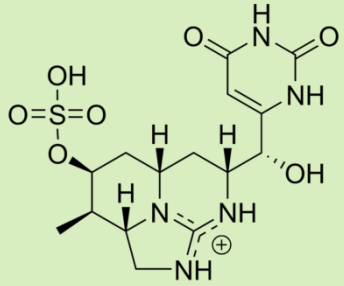




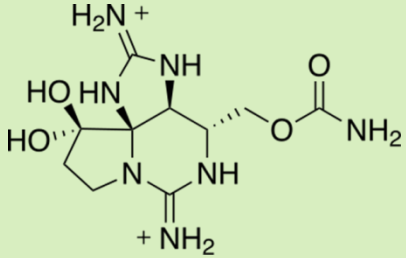




David Crane[1], Cindy Tsai[2] and Abdou Mekebri[2]
[1]CA Dept of Fish and Game (Wildlife) and
[2]San Jose State University Research Foundation
Fish and Wildlife Water Pollution Control Laboratory



SWRCB Cyanotoxin Workshop
Oakland, CA
November 28, 2012



Analytical Challenges

- An area of active research: at least 89 cyanotoxins known to exist[1]
- Need selective and sensitive methods
- Need low cost screening method(s) for large numbers of samples
- Analytical standards exist for only a few toxins
- Toxin-producing genera generally produce more than one cyanotoxin[2]

[1] Walker and Von Dohren, 2006, FEMS Microbiology Reviews, v.30, p. 530-563
[2] Keith Loftin, USGS

Exposure risk and toxin concentration (how low do we need to go?)

WHO risk definitions (*Chorus and Bartram, 1999*):

— Low risk: less than 10 micrograms per liter (µg/L)

— Moderate risk: 10–20 µg/L

— High risk: 20–2,000 µg/L

— Very high risk: greater than 2,000 µg/L

WHO provisional guideline for drinking water

— 1 µg/L for microcystin-LR

Analytical reporting limit needed - (1 µg/L÷10)

— **0.1 µg/L (ppb)**

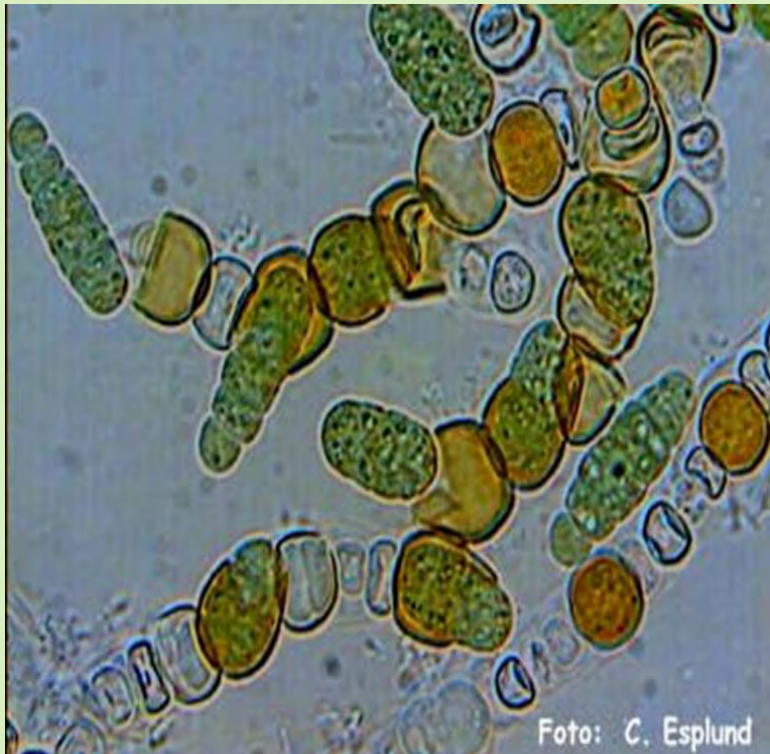
Freshwater Cyanotoxins

Alkaloid toxins:

- Anatoxins (*neurotoxin*)
- Cylindrospermopsins (*hepatotoxin, cytotoxin, neurotoxin*)
- Saxitoxins (*neurotoxin*)

Cyclic peptides:

- Microcystins (*hepatotoxin*)
- Nodularins (*hepatotoxin*)



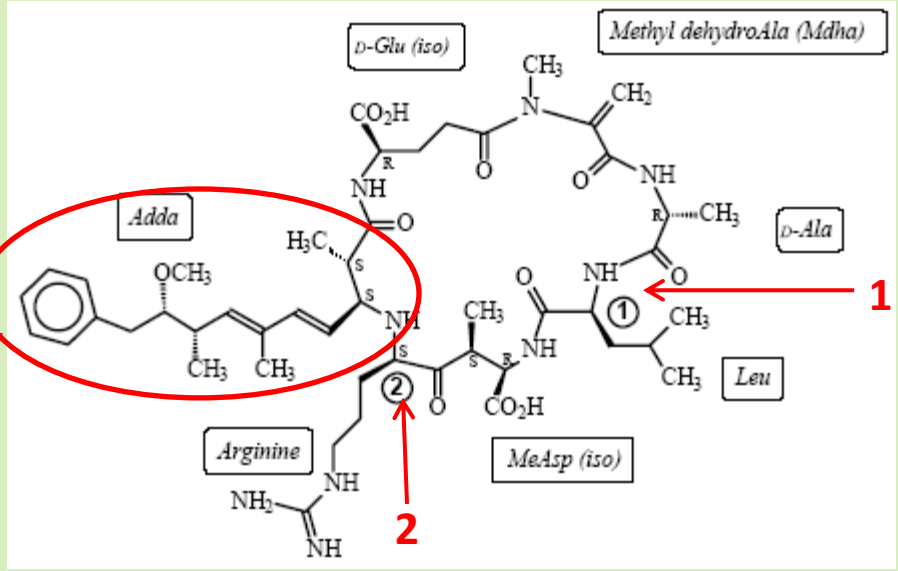
Freshwater Cyanotoxins

Microcystins – cyclic heptapeptides, 7 member ring with 5 non-protein amino acids and 2 protein amino acids which distinguishes the different microcystins (LR,RR,YR,LA,LF,LW,LY) - ***both MCs and nodularin toxins contain the Adda β-amino side group***

	1	2
LR*	Leucine	Arginine
RR	Arginine	Arginine
YR	Tyrosine	Arginine
LA	Leucine	Alanine
LF	Leucine	Phenylalanine
LW	Leucine	Tryptophan
LY	Leucine	Tyrosine

*most common

Adda – β-amino side group



Recommended Sample Handling*

- Toxin samples - processed and shipped same day or within 24 hours @ 4°C stored in the dark (*amber glass, Teflon® or polyethylene*)*
- Toxins may be stored frozen several months or years (*only <u>total</u> toxin concentrations can be measured after freezing*) *
- Toxin LC extracts - analyzed within 40 days

*Cyanobacteria in Lakes and Reservoirs: Toxin and Taste-and-odor Sampling Guidelines (ver. 1.0): USGS Techniques of Water Resources Investigations, Book 9, Chapt A7, Section 7.5, Sep 2008

Cyanotoxin Measurement

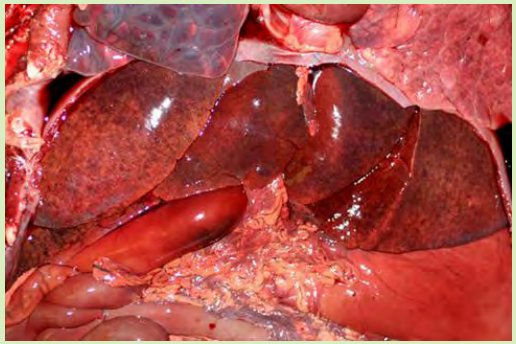
Water and scum:

Total Toxin = Dissolved-phase toxin + particulate/bound toxin (*analysis of total toxin requires **cell-lysis***)

Biological tissues:

Total Toxin = Free toxin + covalently bonded toxin

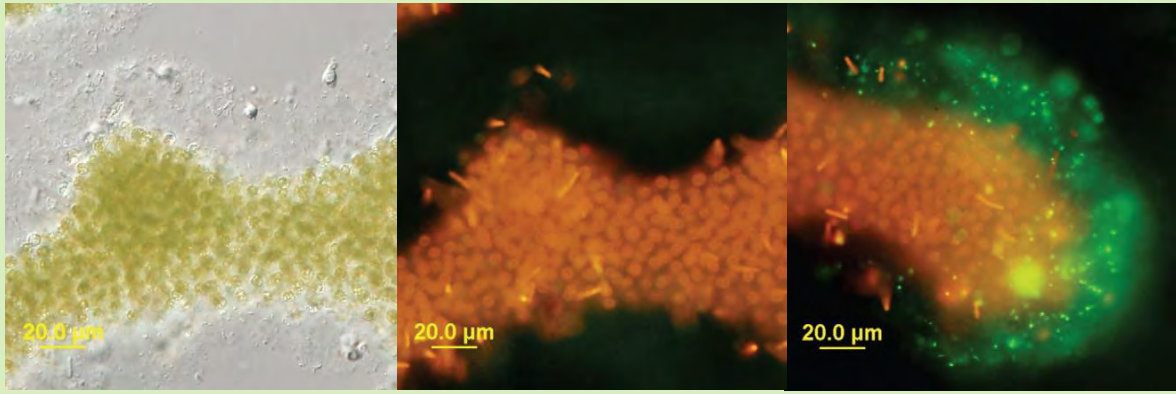
(*Most tissue analysis methods only measure **free** toxin*)



Cell-Lysis Techniques – Eval. Results[1]

- Sonication (at 70 % power-5 min) - ***most effective***
- QuikLyse™ (Abraxis, LLC) – ***least effective***
- Autoclaving, boiling and sequential freeze-thaw treatment – *moderately effective*
- Sequential freeze-thaw – *equal or greater percentage than autoclaving or boiling*
- **Sonication prior to storage may result in loss of toxins[2]**

Sonicated at 70 percent power



Copco Reservoir, CA (9/10/2009). LM-Microcystis aeruginosa. FITC-an orange color dominates the cells. Sytox® green-stain did not penetrate the cell membrane of the cyanobacteria;

[1]B. Rosen, K. Loftin, C. Smith, R. Lane, and S. Keydel (USEPA R9 and USGS 2010)

[2]Mioni et al., Harmful cyanobacteria and their toxins in Clear Lake and The Delta (California), Jan 31, 2012

Biological Tissues - Do we need to know the total (free and covalently bound) MC concentrations? Ans: <u>Maybe</u>

- Bound MC may contribute to the transfer of MC through the food web (*Smith et al. 2010*)
- Covalent complexes are probably not as toxic or are not bioavailable for the next trophic level (*Ibelings and Chorus 2007, Lance et al. 2009*)
- Total MCs in tissues requires additional oxidative digestion
- Probably not to determine exposure

Analysis Methods Available

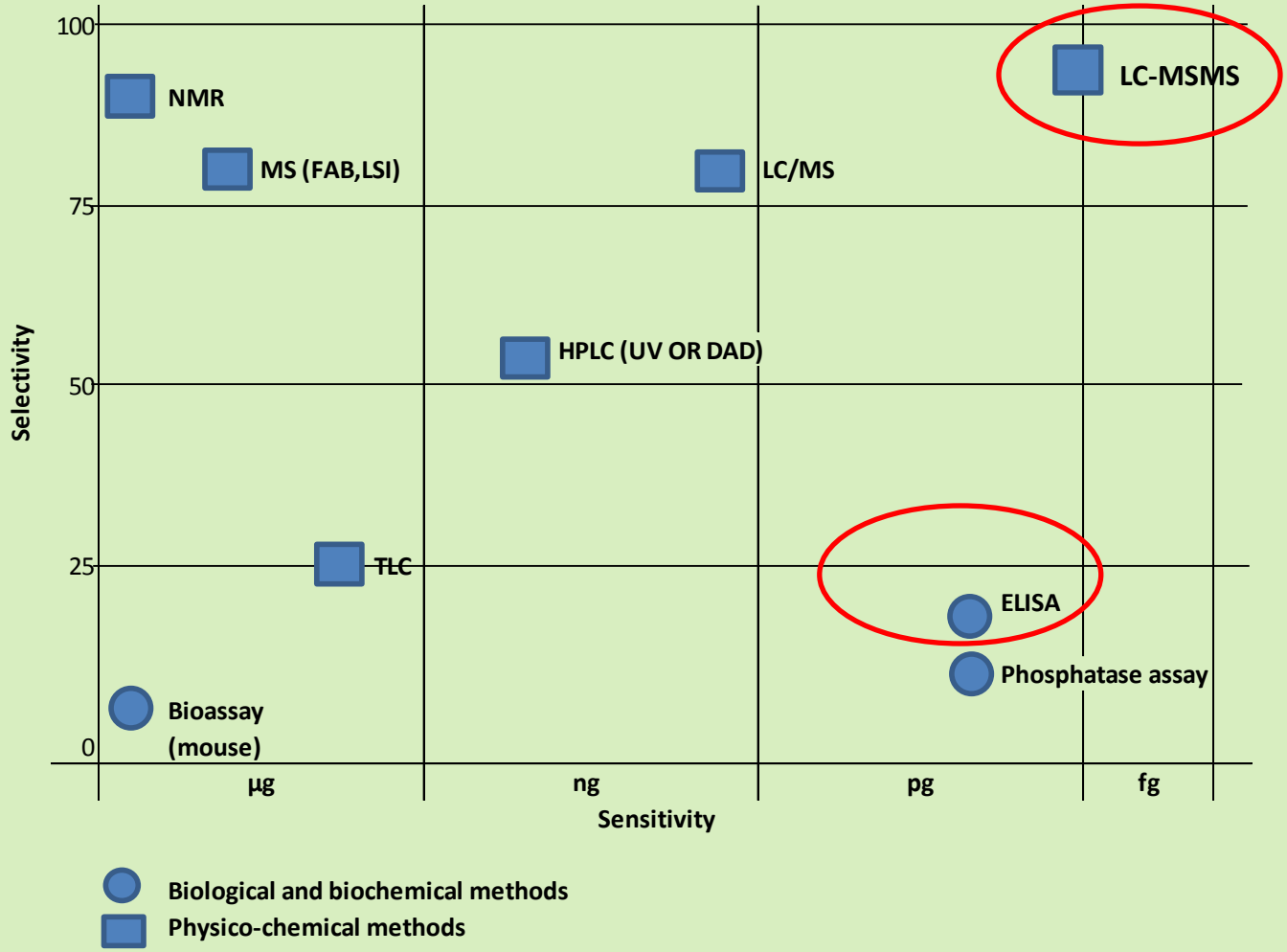
Methods Available for Cyanotoxin Detection

	Freshwater Cyanotoxins				
	Anatoxins	Cylindrospermopsins	Microcystins	Nodularins	Saxitoxins
Biological Assays (Class Specific Methods at Best)					
Mouse	Yes	Yes	Yes	Yes	Yes
PPIA	No	No	Yes	No	No
Neurochemical	Yes	No	No	No	Yes
ELISA	In progress	Yes	Yes	Yes	Yes
Chromatographic Methods (Compound Specific Methods)					
Gas Chromatography					
GC/FID	Yes	No	No	No	No
GC/MS	Yes	No	No	No	No
Liquid Chromatography					
LC/UV (or HPLC)	Yes	Yes	Yes	Yes	Yes
LC/FL	Yes	No	No	No	Yes
Liquid Chromatography combined with mass spectrometry					
LC/IT MS	Yes	Yes	Yes	Yes	Yes
LC/TOF MS	Yes	Yes	Yes	Yes	Yes
LC/MS	Yes	Yes	Yes	Yes	Yes
LC/MS/MS	Yes	Yes	Yes	Yes	Yes

Genetic – Quantitative polymerase Chain Reaction (qPCR) toxin gene identification (future)

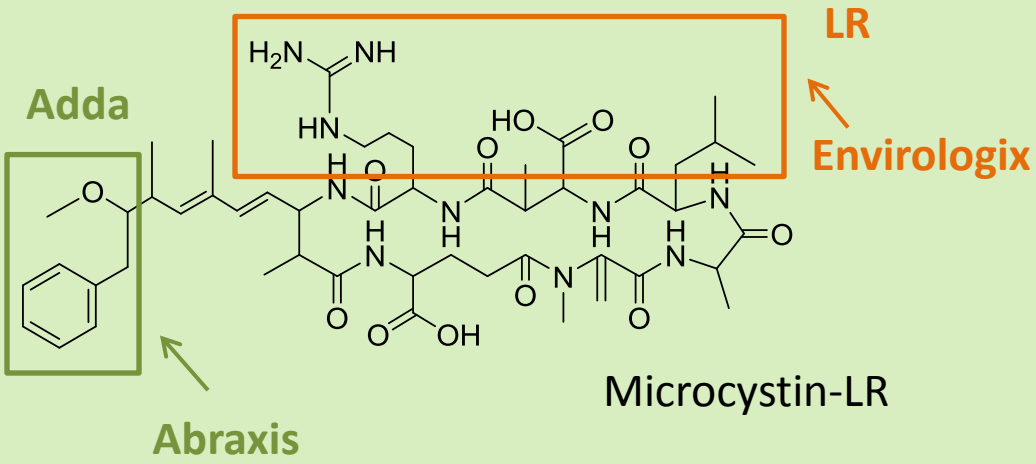
K. Loftin, J. Graham, B. Rosen, USGS 2010

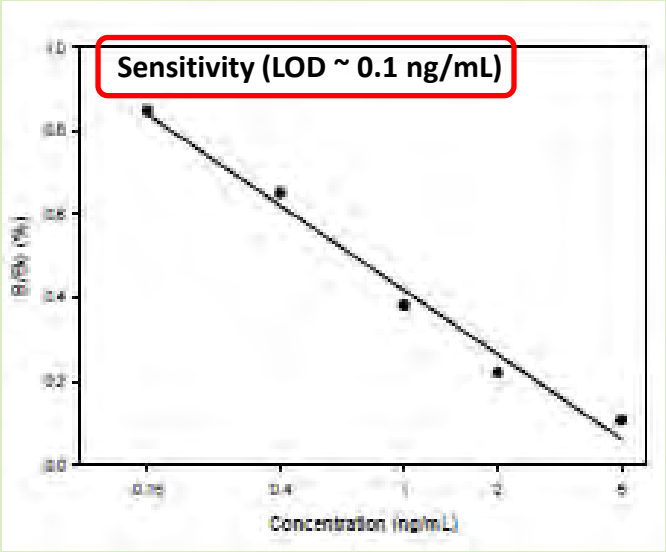
Relationship Between Sensitivity and Selectivity of Analytical Methods for Microcystins*



*Toxic Cyanobacteria in Water: A guide to their public health consequences, monitoring and management, Ch 13, WHO 1999

ELISA kits for microcystins and nodularin

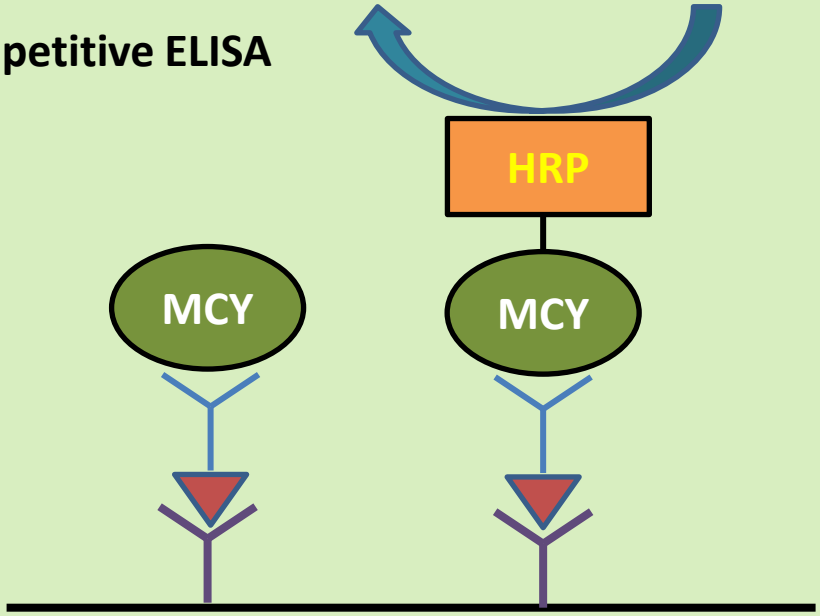




Blue color (450 nm)

Substrate

Competitive ELISA



http://www.abraxiskits.com

ELISA kits for microcystins and nodularin

PROS

- Sensitive for water (0.1 μg/L)
- Inexpensive ($20/sample)
- Good recoveries[1] –
  MC LR kit 73-93%
  %RSD 14-21%
- Analysis doesn't require multiple standards

[1]T. Triantis et al., Toxicon 55 (2010) 979-989.

CONS

- High %rec and RSD - Adda kit (133-189%, %RSD>28%) [1]
- False positives:
  17% MC LR kit and 6% Adda kit[1]
- False negatives:
  15% MC LR kit and **0%** Adda kit[1]
- Variable cross reactivity with other MC variants[1,2,3]
- Matrix interferences (***some severe***)

[1]T. Triantis et al., Toxicon 55 (2010) 979-989.
[2]F. Gurbuz et al. , Environmental Forensics, 13:105-109, 2012
[3]Lawrence et al., JAOAC, 84(4), 2001

ELISA kits for microcystins and nodularin - recommendations

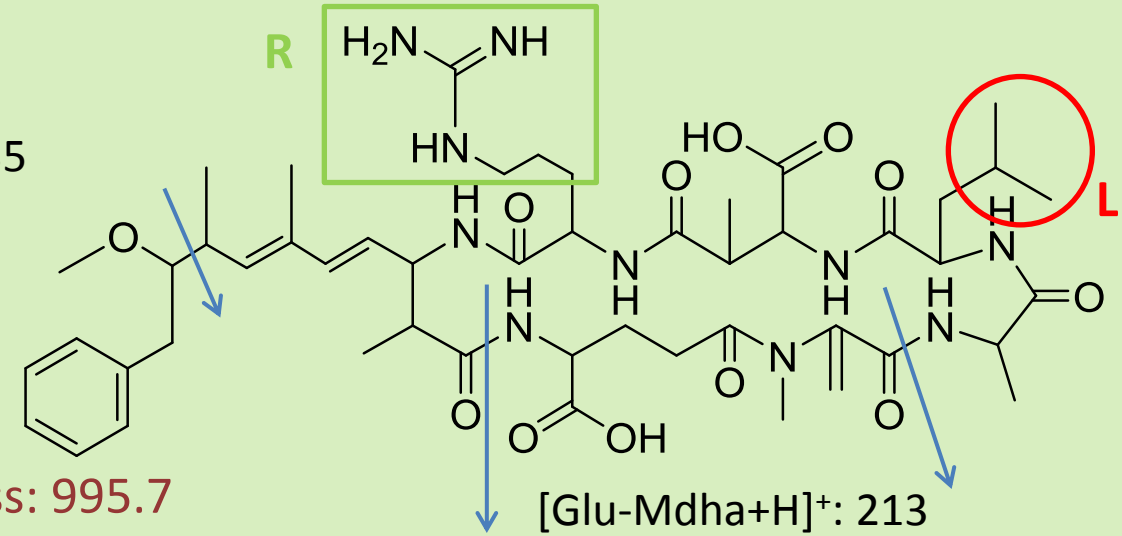
- ELISA kits – should be systematically tested for performance to specific applications including matrix[1]
- Analyst - good technique is important!
- Use of second source standard solutions [1]
- All positive results and a percentage of negative results should be confirmed by LC-MS or LC-MSMS[1]
- LC-MSMS - preferred analysis method for quantitation of MCs (*may agree better with ELISA than LC-MS[2])*

[1] T. Triantis et al., Toxicon 55 (2010) 979-989.
[2] Lawrence et al., JAOAC, 84(4), 2001

Mass spectrometry

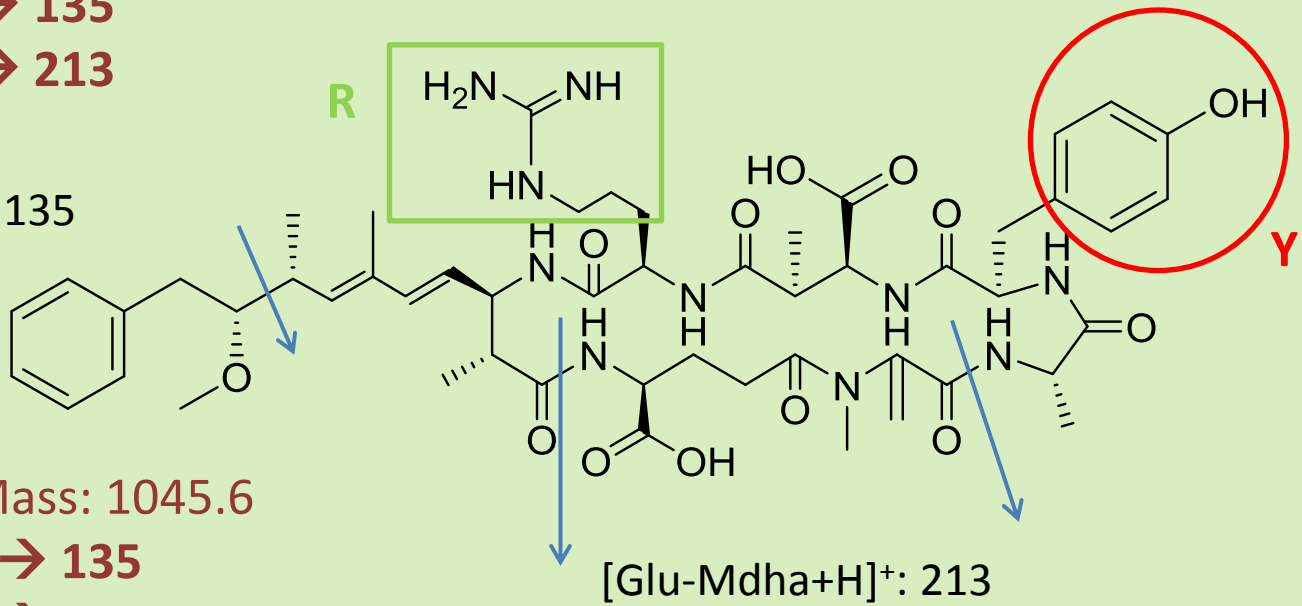
fragmentation of microcystins

[Ph-CH2-CHOMe]$^+$: 135
(Adda group)



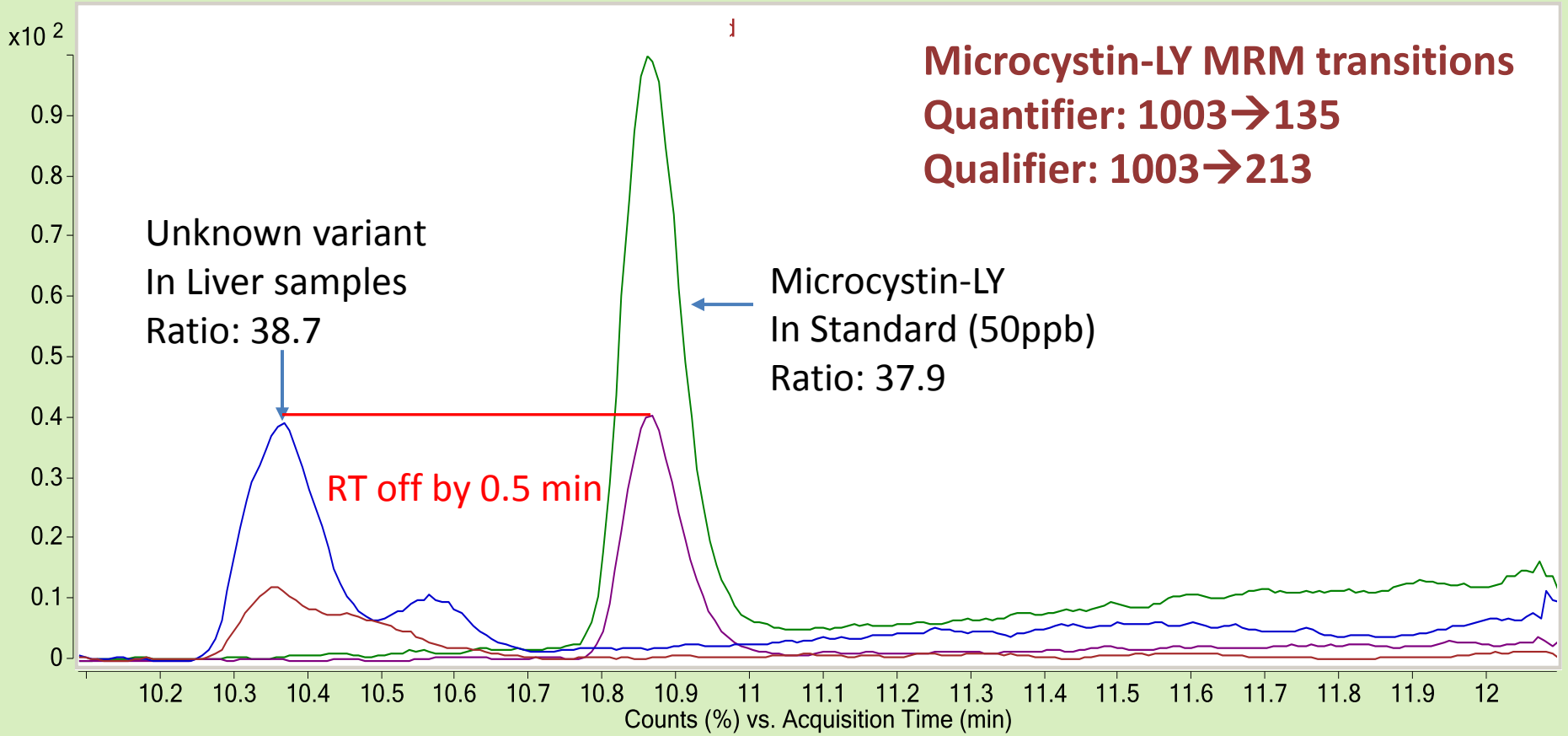
Microcystin-LR Mass: 995.7
Transition - 996 → **135**
Transition - 996 → **213**

[Ph-CH2-CHOMe]$^+$: 135
(Adda group)



Microcystin-YR Mass: 1045.6
Transition - 1046 → **135**
Transition - 1046 → **213**

Unknown microcystin variant in liver- misidentification can occur with MSMS



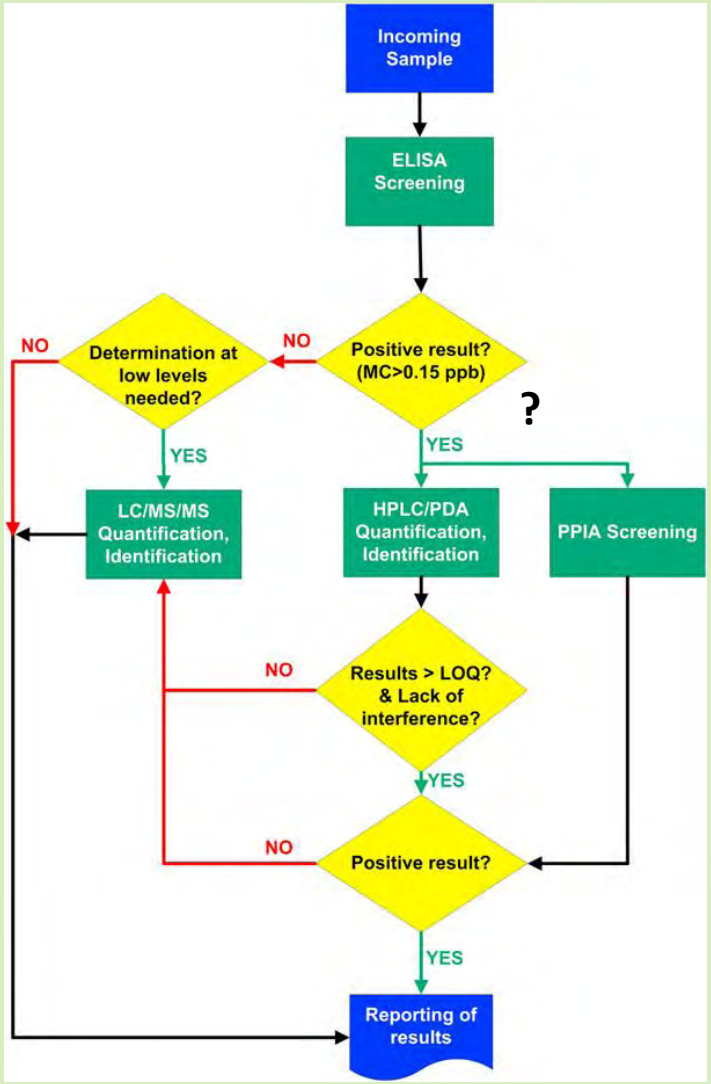
Four criteria must be met for LC-MSMS detection (*RT, quant mass, qual mass, ratio quant/qual*)

LC-MSMS vs ELISA (µg/kg)

Name	Tissue	ELISA-LR Equivalant	LC/MS-MS	Note
L-052-12_1	Muscle	7.15/4.59	ND	
L-052-12_2	Muscle	2.80/1.14	ND	
L-052-12_3	Muscle	3.27/1.58	ND	
L-052-12_4	Muscle	4.07/1.76	ND	
L-052-12_5	Muscle	3.90/2.48	ND	
L-052-12_6	Liver	226/95.4/75.7	ND	Unknown peak for LY
L-052-12_7	Liver	114/66.6	11.9 (LA)	Unknown peak for LY
L-052-12_8	Liver	63.8	9.24/9.42 (LA)	Unknown peak for LY
L-052-12_9	Liver	34.5/152/52.0	2.53 (LA)	Unknown peak for LY
L-052-12_11	Muscle	4.72/2.33	ND	
L-052-12_13	Muscle	16.0/29.1	ND	
L-052-12_14	Muscle	6.75/12.8	ND	
L-052-12_15	Muscle	21.6	ND	
L-052-12_16	Muscle	34.7	ND	
L-052-12_17	Muscle	6.86/13.8/15.0	ND	

Protocol for monitoring MCs in water

T. Triantis et al./Toxicon 55 (2010) 979-989



- All pos ELISA samples quantified by 2nd (HPLC) meth
- 20% of ELISA pos samples confirmed by LC-MSMS
- 5% of ELISA neg samples confirmed by LC-MSMS

Analysis costs

- PPIA (lowest)
- ELISA (30-50% lower than HPLC)
- HPLC (40% lower than HPLC-MSMS)
- HPLC-MS (close to cost of MSMS)
- HPLC-MSMS (highest)

Anticipated advantages

1. Lower lab costs for large scale monitoring
2. ELISA screening provides fast results for large numbers of samples
3. Significant savings anticipated even if all ELISA positive samples are confirmed by LC-MSMS

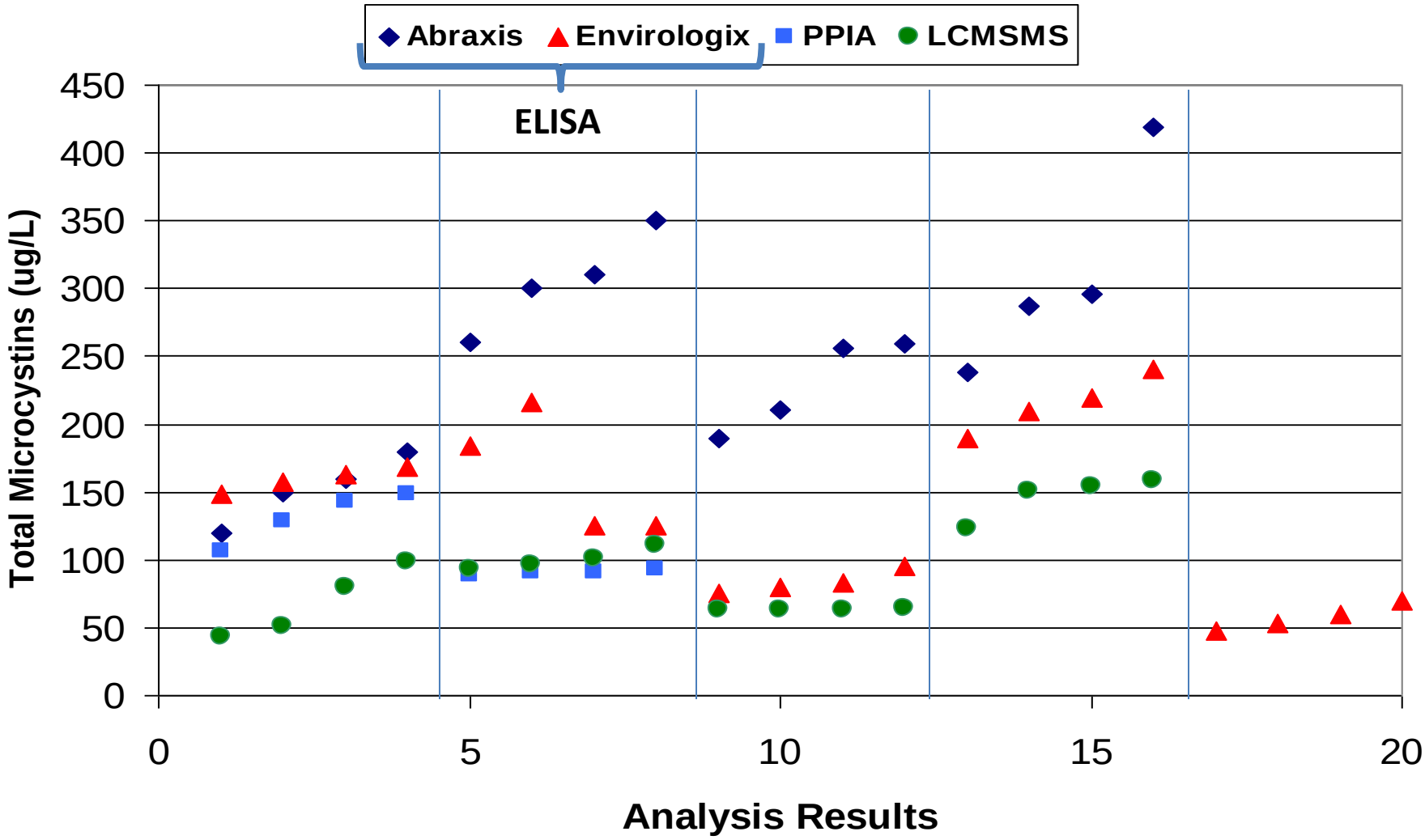
Florida Department of Environmental Protection Round Robin - 2007

DFG participation in nation-wide round robin exercise with eleven laboratories for the analysis of microcystins by ELISA, PPIA and LC/MS/MS

Ten water samples:

- 3 Replicates FDEP standard
- 3 Replicates University of Texas culture
- 4 Replicates Lake Munson sample

FDEP Round Robin - Lake Munson Culture Results



Current DFG Lab Capabilities

Cyanotoxin analytes currently reported (*LC-MSMS*):

- MC (*RR, dmRR, LR, dmLR, YR, LA, LF, LW and LY*)
- Anatoxin a
- Nodularin

Cyanotoxin analyte future additions (requires different extraction method and LC column):

- BMAA (*β-N-methylamino-L-alanine*)
- Saxitoxin (*requires validation*)
- Cylindrospermopsin (*requires validation*)

Current DFG lab MDLs/RLs for water

Cyanotoxin	MDL (ug/L)	RL (ug/L)
MC RR	0.009	0.020
MC dmRR	0.010 (est)	0.020
MC LR	0.005	0.020
MC dmLR	0.010 (est)	0.020
MC YR	0.015	0.020
MC LA	0.013	0.020
MC LF	0.020	0.020
MC LW	0.024	0.020
MC LY	0.010 (est)	0.020
Anatoxin a	0.050	0.100
Nodularin	0.009	0.020

Current DFG lab MDLs/RLs for tissue

Cyanotoxin	MDL (ng/g)	RL (ng/g)
MC RR	0.500	1.00
MC dmRR	0.500 (est)	1.00
MC LR	0.500	1.00
MC dmLR	0.500 (est)	1.00
MC YR	0.500	1.00
MC LA	0.500	1.00
MC LF	0.500	1.00
MC LW	0.500	1.00
MC LY	0.500 (est)	1.00
Anatoxin a	5.00	10.0
Nodularin	5.00	10.0

Summary

- There is no perfect analysis method
- Screening with ELISA followed by quantitative confirmation by LC-MSMS is a good approach
- (5%?) of ELISA negative results should be confirmed by LC-(DAD, MS, or MSMS)
- Future routine use of polymerase chain reaction (qPCR) to determine if potentially toxic organisms are present
- Clear communication w/laboratories required to ensure relevant results (*always the case!*)


Thanks for Listening