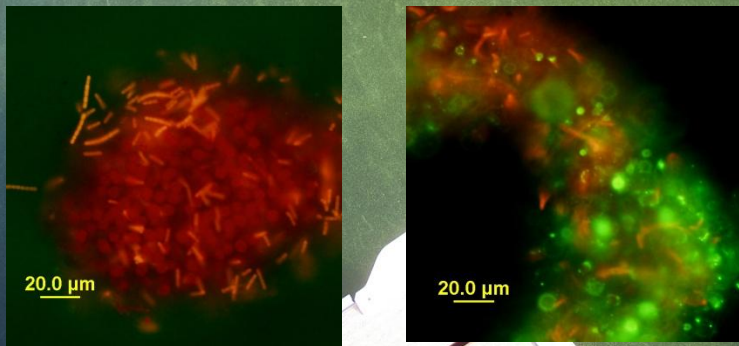


# Analytical Methods for Cyanotoxin Detection and Impacts on Data Interpretation

Irongate Reservoir, CA  
Dominated by *Microcystis* sp.



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Ann St. Amand, Phycotech

Workshop  
Guidelines for Design, Sampling, Analysis and Interpretation for Cyanobacterial Toxin Studies  
2010 National Water Quality Monitoring Conference  
Denver, CO, USA

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# Cyanotoxin Measurement Background

Analytical measurements, regardless of techniques, are only surrogates for actual toxicity!

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Organisms of concern dictate which exposure routes and environmental compartments we are focused on. This of course dictates if a sample needs to be split in the laboratory and how it should be processed.

This then dictates whether we need to process the sample from the standpoint of intracellular, extracellular, total toxin concentrations, or some combination.

Sample splitting in the field and laboratory are important for collection of QA/QC field and laboratory data and also when we are interested in tracking a combination of intracellular, extracellular (dissolved or bound), and/or total toxin concentrations.

We want robust, reproducible, accurate, and precise analytical methods with adequate sensitivity (method reporting limits at least 10 times lower than our toxicological threshold concentrations).

# Exposure Routes Versus Sample Processing

There are several questions that need to be answered prior study design:

1. What organisms are we concerned with?
2. What is the duration of exposure?
3. What environmental compartments do we need to sample?
4. What hypotheses or monitoring efforts are we attempting to address?

Exposure Routes include:

Consumption

Water

Food

Algal Supplements

Respiration

Inhalation in terrestrial fauna

Gills or other breathing apparatus in aquatic species

Direct introduction

Dialysis

Open wounds

Dermal (allergic responses, rashes, etc.)

Plant uptake

Sample Processing:

**Total toxin results** will generally measure the worst case scenario for any type of exposure.

**Dissolved-phase toxin results** will be quite variable depending on cyanobacteria age, environmental stress (e.g. nutrient levels, light intensity, climate, etc.) and can range from non-detect for healthy cells to equivalent to total toxin results for completely lysed cells.

**Particulate-phase toxin** will generally include toxins sorbed to organic and inorganic material. This phase can also include toxin from unlysed cyanobacteria, but is dependent upon how the sample is processed (e.g. Is lysis used and how effective is it).

**Bound toxin** is generally discussed in terms of tissues. Toxins can be reversible or irreversibly bound.



Grand Lake (Lake St. Mary), OH  
Drinking water source, recreation, food source

Photo courtesy of Linda Mason-Merchantbrink, OH EPA

# How Do We Define the Analytical Process for Cyanotoxin Measurement?

There are a wide variety of techniques and analytical methods used in each step of the process. Each step effects the other, so study design, data quality, and interpretation become an iterative process.

## Study Design and Sample Collection

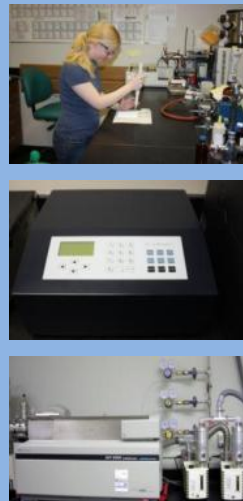


## Laboratory Processing

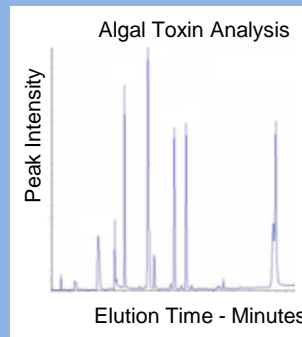


## The Laboratory

### Analysis

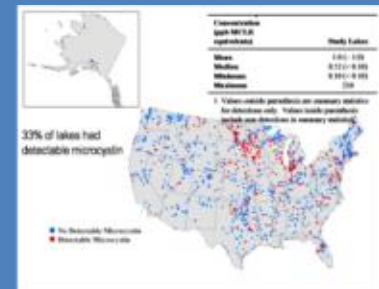


### Data Reduction And Laboratory QA/QC



## Study Results

### Interpretation And Project QA/QC



# Cyanotoxin Measurement Background

Total Toxin = Extracellular Toxin (dissolved and bound) + Intracellular Toxin



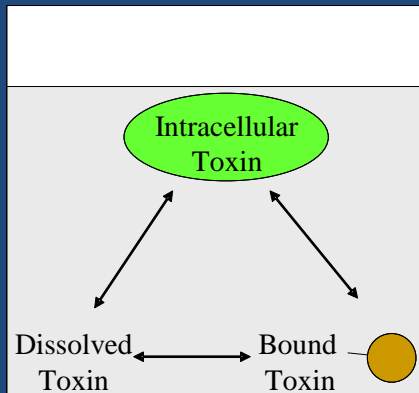
Total Toxin



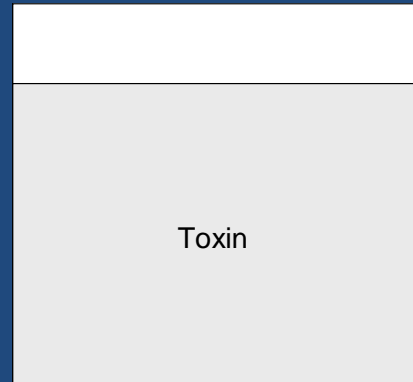
Dissolved-Phase Toxin (Extracellular)



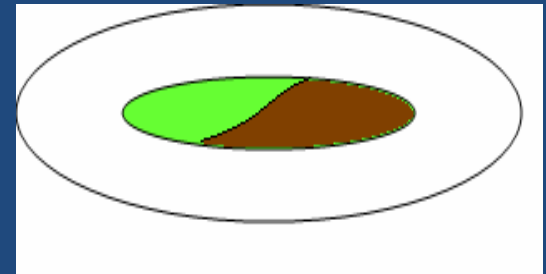
Particulate / Bound Toxin – meaning depends on whether cells are lysed.



=



+



# Cyanobacteria Cell Structure: A Tough Nut To Crack...

Current thought indicates that cyanotoxins are contained in the cytoplasm of intact cyanobacteria (intracellular) and that toxins are released to the surrounding environment (extracellular - dissolved or bound) upon cell membrane disruption by natural (e.g. senescence, apoptosis, etc.), in source treatment (algicides) or laboratory processes.

Cyanobacterial cell structure is not as well understood as for other bacteria.

However, we do know that:

Cyanobacteria have three membranes:

1. Thylakoid membrane –where photosynthesis and respiration occur.
2. Plasma membrane
3. Outer membrane

Some cyanobacteria also have an S-layer and/or a mucilaginous sheath of varying degrees of thickness.

Based on existing research scientists think:

Cyanobacteria do not have membrane bound Organelles (e.g. no nucleus therefore genetic material is believed to be in the cytoplasm).

Cyanotoxins are also believed to be stored in the cytoplasm.

## Generic Cyanobacteria Cell

Mucilaginous Sheath (Polysaccharide-Based)

S-Layer (or Surface Layer, Protein-Based: paracrystalline, porous) - Not found in all cyanobacteria

Outer Membrane (Lipopolysaccharide-Based, proteins, porins present)

Periplasmic Space

Peptidoglycan Layer

Plasma Membrane (or Cytoplasmic Membrane)

Thylakoid Membrane

Photosystem I

Photosystem II



DNA / RNA

Toxins



Cytoplasm

Toxins



DNA / RNA

Cyanobacteria are oxygenic prokaryotic bacteria and are different from other prokaryotes due to the presence of chlorophyll for photosynthesis.



Age and stress also play a role in cell structure!

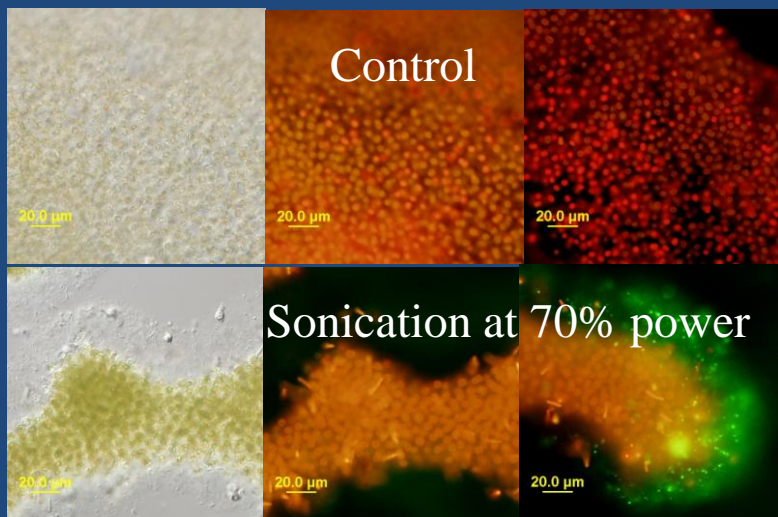


# Multiple Laboratory Cell-Lysis Techniques (Total Toxin)

Variations of one of 6 techniques are routinely used:

1. Sequential Freeze/Thaw Cycles
2. Sonication
3. Freeze Drying
4. Boiling
5. Autoclave
6. Chemical/enzymatic (e.g. solvents, enzymes, etc.)

*Microcystis* from Copco Reservoir, OR



Images courtesy of Barry Rosen, USGS

# What Methods Are Available For Cyanotoxin Measurement?

## Biological Assays:

Animal Tests (e.g. Mice)

Enzyme-Linked Immunosorbent Assays (ELISA)

Protein Phosphatase Inhibition Assays (PPIA)

Neurochemical assays (e.g. acetylcholinesterase-based)



## Chromatographic Methods:

Gas Chromatography with Flame Ionization Detection (GC/FID) or Mass Spectrometry (GC/MS)

Thin Layer Chromatography (TLC)

Liquid Chromatography / Ultraviolet-Visible Detection (HPLC or LC/UV)

Liquid Chromatography / Fluorescence (LC/FL) – usually with post column oxidation prior to detection

Liquid Chromatography Ion Trap Mass Spectrometry (LC/IT MS)

Liquid Chromatography Time-of-Flight Mass Spectrometry (LC/TOF MS)

Liquid Chromatography Single Quadrupole Mass Spectrometry (LC/MS)

Liquid Chromatography Triple Quadrupole Mass Spectrometry (LC/MS)





# What Methods Are Available For Cyanotoxin Measurement?

	Freshwater Cyanotoxins				
	Anatoxins	Cylindrospermopsins	Microcystins	Nodularins	Saxitoxins
<b>Biological Assays (Class Specific Methods at Best):</b>					
Mouse	Y	Y	Y	Y	Y
PPIA	N	N	Y	N	N
Neurochemical	Y	N	N	N	Y
ELISA	?	Y	Y	Y	Y
<b>Chromatographic Methods (Compound Specific Methods):</b>					
<i>Gas Chromatography:</i>					
GC/FID	Y	N	N	N	N
GC/MS	Y	N	N	N	N
<i>Liquid Chromatography:</i>					
LC/UV (or HPLC)	Y	Y	Y	Y	Y
LC/FL	Y	N	N	N	Y
<i>Liquid chromatography combined with mass spectrometry can analyze cyanotoxins very specifically.</i>					
LC/IT MS	Y	Y	Y	Y	Y
LC/TOF MS	Y	Y	Y	Y	Y
LC/MS	Y	Y	Y	Y	Y
LC/MS/MS	Y	Y	Y	Y	Y

Y = Yes

N = No

? = It is being worked on

# Method Specificity for Cyanotoxins

## Specificity

### Biological Assays (Class Specific Methods at Best):

Mouse	Non-specific, test must be rapid therefore endpoint usually death.
PPIA	Of the freshwater cyanotoxins, only microcystins are known to inhibit protein phosphatase.
Neurochemical	Of the freshwater cyanotoxins, only anatoxins and saxitoxins are known to inhibit neurochemical processes.
ELISA	Compound and toxin class specificity dependent on antibody or mix of antibodies used.

### Chromatographic Methods (Compound Specific Methods):

#### *Gas Chromatography:*

GC/FID	Only the anatoxins have been routinely measured. Derivatization is typically required.
GC/MS	Only the anatoxins have been routinely measured. Derivatization is typically required.

#### *Liquid Chromatography:*

LC/UV (or HPLC)	Variable. Subject to interference with co-eluting matrix.
LC/FL	Variable. Subject to interference with co-eluting matrix.

#### *Liquid chromatography combined with mass spectrometry can analyze cyanotoxins very specifically.*

LC/IT MS	Second in compound specificity only to LC/TOF MS.
LC/TOF MS	Accurate mass capability makes this technique the most specific.
LC/MS	Weaker cousin of LC/MS/MS. Fourth most specific.
LC/MS/MS	Third most specific technique routinely employed

# Taste-and-Odor Detection

Geosmin and 2-methylisoborneol (MIB) are the two most commonly measured taste-and-odor compounds.

As semi-volatiles, they are commonly measured by GC/MS.

Rapid assessment tools (e.g. ELISAs) are on the horizon and will be a welcome addition.



# Quality Assurance / Quality Control (QA/QC) for Analytical Data

**Field:** This is typically one of the most overlooked areas in study design! This needs to be decided before we go to the field to collect samples.

**1. Blanks** – this indicates that your sampling equipment was uncontaminated prior to sampling.

**2. Split Sample Replicates** – This type of sample replicate is aimed at evaluating the laboratory precision, however sample splitting of cyanobacteria is problematic to say the least. Keep in mind that this is also an evaluation of the sample splitting technique.

**3. Concurrent/Sequential Sample Replicates** – This type of sample replicate is aimed at evaluating environmental and field sampling variability.

**3. Spiked Sample Duplicates** – Spiked sample duplicates can indicate analyte losses (or recovery) in the field.

Careful consideration must be used regarding when, where, and if to do spikes because of potential degradation of the spiked analyte, possible confusion in sample labeling (false positives), risk contaminating sample collection equipment, etc. Interpretation will be dependent on where the sample is spiked in the field sampling process.

**Laboratory:** QA/QC should be designed to address variability from sample processing and analysis!

**1. Blanks** indicate that the laboratory dilution water is uncontaminated with the analyte of interest.

**2. Spiked Blanks** indicate that laboratory dilution water is not causing matrix effects and indicates the degree of agreement between the spiked standard and the calibration curve. This is important for interpreting spiked sample duplicates to differentiate between matrix effects and incorrect spiked standard concentration.

**3. Sample Duplicates** – This indicates how reproducible the laboratory sample processing/analysis component is.

**4. Spiked Sample Duplicates** – indicates the presence of matrix effects in sample analysis or recovery losses if samples are spiked before sample processing.

# Quantitation of Enzyme Linked Immunosorbent Assay (ELISA) Data

## Calibration Curves:

The choice of calibration curve can impact both detection frequency and concentration. Be consistent!

Historically, Semi-log fits have been used to a great extent.

With modern computing power, a four-parameter curve fit is much more common and robust, typically.

- We need at least five calibration levels since there are four unknowns in this equation.
- This curve fit can be done by using Microsoft Excel<sup>®</sup> if the ELISA reader software is not capable.

Calibration curves for a class of compounds are typically quantitated using the response curve of a single compound (e.g. microcystin-LR).

## Cross-reactivity :

ELISAs typically react with several analytes within a given chemical class. Each ELISA has a unique response to a range of analytes that an assay is reactive with based on the antibodies used. In other words, the responsiveness of microcystin-LR is likely not equal to microcystin-LA, etc.

An ELISAs cross-reactivity pattern coupled with the analytes present can impact whether a measured value under or over reports actual concentrations.

## Data:

Method Reporting Limits - Standard practices suggest that data should not be report below the lowest calibration standard. This should not be confused with method detection limit which is frequently reported by manufacturers below the lowest calibration standard based on the linear portion of the calibration curve or precision at a certain standard concentration.



For more information on ELISA see: *Immunoassay and other Bioanalytical Techniques*, Van Emon, J.M., Ed.; CRC Press, 2007.

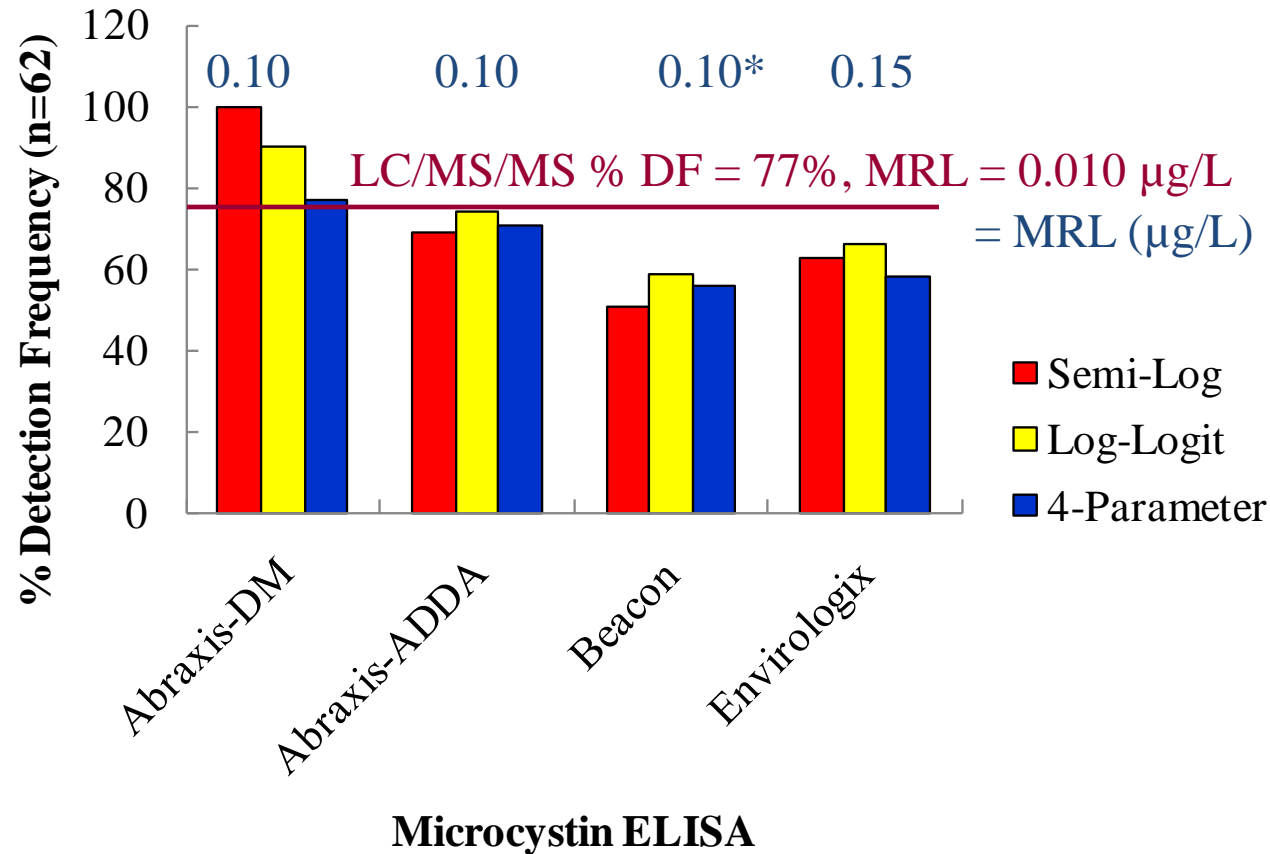
# ELISA Calibration Curve Fitting Equations Can Impact Detection Frequency for Environmental Samples

- The Abraxis-DM ELISA exhibited the largest spread when comparing detection frequency between curve fits (77 to 100% - %RSD=13%).

- The Abraxis-ADDA ELISA appeared to be the least sensitive to curve fit based on detection frequency.

- The Abraxis-ADDA and Abraxis-DM ELISA (4-Parameter) appeared to agree most closely with LC/MS/MS given the 8 congeners measured and a ten times lower MRL.

- The Strategic Diagnostics assay did not meet QA/QC criteria for precision and accuracy for control standards and replicate analyses.



\*Beacon does not have a manufacturer derived MRL. 0.10 used for Beacon MRL since low standard was 0.10 µg/L.

# ELISA Cross-Reactivity Example for Microcystins

No ELISA is MCLR specific!

- With over 80+ microcystin and 10+ nodularins, most cross-reactivities are unknown!

Microcystin Assays	Percent Cross-Reactivity							
	MCLA	MCLF	MCLR	MCRR	MCLW	MCLY	MCYR	NODR
<i>Monoclonal Assays</i>								
Abraxis-DM	48	72	100	53	102	NA	64	76
<i>Polyclonal Assays</i>								
Abraxis-ADDA	125	108	100	91	114	NA	81	169
Beacon	5	NA	100	87	NA	NA	48	31
Envirologix	62	NA	100	54	NA	NA	35	69
Strategic Diagnostics	23	NA	100	97	NA	NA	82	66

ELISA Response =  $\Sigma$  (Cross-Reactivity x Actual Congener Concentration) ,

Example: Theoretical Concentration: 1 ppb MCLR + 1 ppb MCLA = 2  $\mu\text{g/L}$

Abraxis-DM  $\approx 1.48 \mu\text{g/L}$

Abraxis-ADDA  $\approx 2.25 \mu\text{g/L}$

Beacon  $\approx 1.05 \mu\text{g/L}$

Envirologix  $\approx 1.62 \mu\text{g/L}$

SDI  $\approx 1.23 \mu\text{g/L}$

# Quantitation of Liquid Chromatography Mass Spectrometry Data

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## Sample Concentration / Sample Cleanup

Sample concentration and cleanup may be needed depending on method sensitivity and matrix effects. This adds another layer of complexity and expense to analyses.

Solid-phase extraction (SPE) has been the technique of choice for environmental sample concentration / cleanup for over two decades. However, limited success has been observed using SPE on cyanotoxins. Variability may be related to chemistry, but could also be a function of ineffective sample splitting for comparisons.

## Quantitation:

The choice of quantitation technique is largely dependent on matrix effects (e.g. how dirty the sample is, etc.) and availability of standards and isotopes of sufficient quantity and quality. Cost and availability of standards are still an issue.

Quantitation is typically based on the response of the analyte of interest compared to ELISA which uses a surrogate (e.g. microcystin-LR for microcystin-LA).

External calibration curve – Technically this technique is inferior to other techniques.

Internal calibration curve – If matrix effects are not pronounced and a good internal standard is available, this is the cheapest and most effective option.

Standard addition – When matrix effects are present and isotopically labeled standards are not available, this is the technique of choice.

Isotope dilution – When matrix effects are present, this technique will provide equivalent or better data quality than standard addition.

## Data:

There are a range of methods for calculating and dealing with method detection levels (MDLs), method reporting levels (MRLs), and non-detections.



# Summary

Clearly define your project objectives, hypotheses, organisms of interest, and final data quality needed before study design initiation.

Don't forget to include sample collection for field and laboratory QA/QC samples!

Sample splitting techniques have not been well characterized to date, but preliminary data indicates this may be a source of much variability in the final data value.

There are a lot of laboratory sample processing techniques and analytical techniques available to measure cyanotoxins. ELISAs have emerged as one of the most common techniques to screen for cyanotoxins. No single approach has emerged as the single best way to process samples and measure cyanotoxins.

Liquid chromatography coupled with mass spectrometry is the preferred platform for identification and quantitation of individual cyanotoxins.

Analyte specific methods tend to be more expensive and require greater expertise.

Pick an approach and use it because continuity is key to understanding any trends in data, but make sure that the approach will generate data of sufficient quality to answer your questions.

Laboratory and analytical techniques will continue to evolve as we learn more about cyanobacteria cell structure, cyanotoxin production, and sources of variability (error).

Spring Lake, CA  
*Microcystis sp. and Woronichinia sp.*



Photo courtesy of Sue Keydel, US EPA

# Ongoing and Completed USGS Methods Related Studies

## Ongoing Studies:

Evaluation of Laboratory Methods of Cyanobacterial Cell Lysis for Microcystin Recovery and Solid Phase Extraction, US EPA Regional Methods Program

Evaluation of Field Techniques for Sample Splitting of Cyanobacterial Bloom Samples, USGS

Continued Expansion of Direct Injection LC/MS/MS Method For Multiple Classes Of Algal Toxins

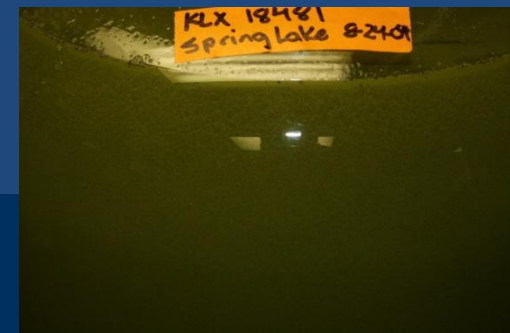
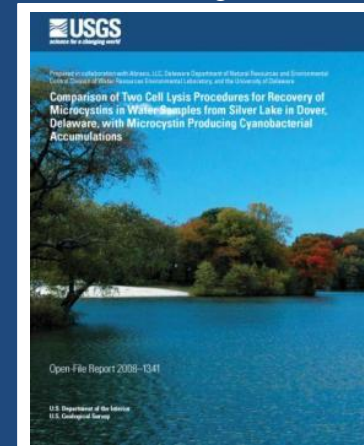
Screening of Environmental Samples By LC/TOF-MS For Unknown Algal Toxins

Verification of Microcystin ELISA Test Kits, US EPA ETV Program, in collaboration with Batelle

## Completed Studies:

Evaluation and Comparison of Microcystin Analysis Using Five Commercial Enzyme-Linked Immunosorbent Assays and Liquid Chromatography Triple Quadrupole Mass Spectrometry, journal manuscript under consideration by *Analytical Chemistry*.

*Comparison of Two Cell Lysis Procedures for Recovery of Microcystins in Water Samples from Silver Lake in Dover, Delaware, with Microcystin Producing Cyanobacterial Accumulations*; U.S. Geol. Sur., Open-File Rep. 2008-1341, 2008 <http://pubs.usgs.gov/of/2008/1341/>



# Resources

Immunoassay and other Bioanalytical Techniques, Van Emon, J.M., Ed.; CRC Press, 2007.

*Comparison of Two Cell Lysis Procedures for Recovery of Microcystins in Water Samples from Silver Lake in Dover, Delaware, with Microcystin Producing Cyanobacterial Accumulations*; U.S. Geol. Sur., Open-File Rep. 2008-1341, 2008

<http://pubs.usgs.gov/of/2008/1341/>

**SIR 2008-5038** *Guidelines for Design and Sampling for Cyanobacterial Toxin and Taste-and-Odor Studies in Lakes and Reservoirs*

<http://pubs.usgs.gov/sir/2008/5038>

**USGS National Field Manual Chapter 7.5** *Cyanobacteria in Lakes and Reservoirs: Toxin and Taste-and-Odor Sampling Guidelines*

<http://water.usgs.gov/owq/FieldManual/Chapter7/7.5.html>

USGS Kansas Algal Toxin Research Team: <http://ks.water.usgs.gov/studies/qw/cyanobacteria/>

USGS Microbiology Water Quality and Cyanobacteria: [http://microbiology.usgs.gov/water\\_quality\\_cyanobacteria.html](http://microbiology.usgs.gov/water_quality_cyanobacteria.html)

*The Cyanobacteria: Molecular Biology, Genomics, and Evolution*, Herrero, A., Flores, E., Eds.; Caister Academic Press, 2008.

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St. John's River, FL  
*Microcystis* sp.

Photo courtesy of Barry Rosen, USGS