EPA United States Environmental Protection Agency

Cyanotoxins Analytical Methods Overview and New Developments

EPA's CyanoSymposium – October 18, 2023

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The views expressed in this presentation are those of the author and do not necessarily represent the views or policies of the U.S. Environmental Protection Agency.

Outline

- Field Sampling Workflows- Recap
- Analytical Methods Overview
- EPA's Published Cyanotoxins Analytical Methods for Drinking and Ambient Waters
- Status of Known Fish Tissue Methods
- Benthic Methods Research Project
- Laboratories Lists– Methods Capabilities
- Future Directions
- EPA Region 10 Analytical Methods Capabilities and New Advances
- Resource List

Monitoring Methods Considerations

- Are there strong prevailing winds or flows?
  - Is it downwind/pushed to shoreline?
- Is it a strongly stratified water with a deep chlorophyll or phycocyanin maximum?
- Is there past data on seasonal or interannual variability in dominant organisms and toxins?
- Are there known nutrient, temperature, or other water quality issues?
  - Are they localized or diffuse?
- Is the bloom primarily benthic, planktonic, or both?
- Are public water system intake impacts a concern or primarily recreation? Other water uses of concern?

Example CyanoHABs Monitoring Workflow

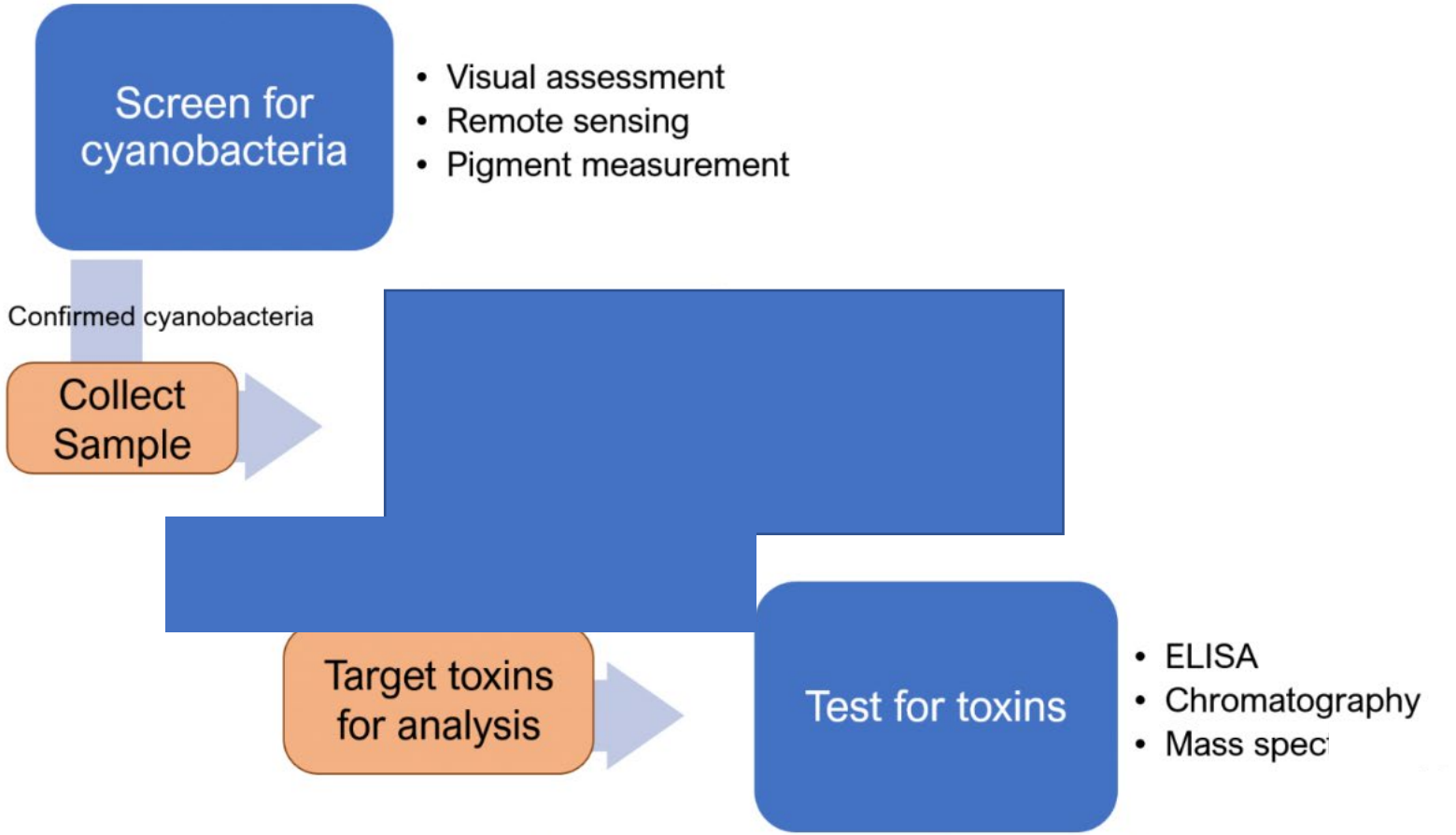

Figure 4-3. Common sequence of monitoring steps used to evaluate risk from cyanobacteria exposure. Your cyanobacteria response plan outlines how analytical data are used to determine next steps (see Figure 5.2, Idaho's HCB Response Plan)

Modified from ITRC 2022; based on Idaho's HCB Response Plan

Initial Visual Observation Data Collection

Initial detection of freshwater harmful algal bloom (HAB) events relies on qualitative, visual observations of blooms formation, including:

- surface water discoloration (e.g., a red, green, or brown tint)
- thick, mat-like accumulations on the shoreline and surface
- fish kills
- probe (sonde) data
- remote sensing tools
    - EPA developed the Cyanobacteria Assessment Network (CyAN) Mobile and Web Application for the early detection of algal blooms in U.S waters. *EPA's Blake Schaeffer will present on remote sensing tools on CyanoSymposium 2023 Day 3.*

Early Warning and Screening Methods

- Sondes – phycocyanin and chlorophyll fluorescence; turbidity, pH and DO variability
- Microscopy/density determinations
- Flow cytometry
- Solid-phase adsorption tracking tools (SPATTS)
- qPCR- DNA and RNA analyses
- Cyanotoxin screening methods
- Remote sensing [will be covered in Day 3, Blake Schaeffer's talk]

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- Cyanotoxin qualitative and quantitative methods
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Passive Samplers

- Solid-phase adsorption tracking tools (SPATTS) are truly passive, samplers. They provide semi-quantitative results (e.g., ng cyanotoxins/gram resin)
- Extraction typically in methanol and then dried; similar analytical procedure after that as water samples
- USGS SPATT samplers deployed in the Oregon Cascades have shown detectable concentrations of atx-a, mcs, cyl, and stx. (Carpenter 2023)
- Sorb extracellular toxins
- Need to be constantly inundated and most can be deployed for a week or two, maximum.
- Other passive samplers (e.g., o-DGT membrane based passive sampler) may be more quantitative, but also more expensive and harder to deploy.

eDNA/eRNA

Environmental DNA/RNA via qPCR: require primers, extraction kit, and thermocycler and clean techniques of collection and analysis

- The steps in performing genetic assays typically include filtration (centrifugation), cell lysis, and extraction of genetic material; adding enzymes, primers, deoxynucleotide triphosphates, and a buffer (available via commercial kits); and, finally, amplifying gene targets and detecting via a thermal cycler. Samples are typically run in a laboratory, but there are new field instruments.
- 16s rRNA can be used for community diversity analysis
- Metagenomics can look broadly at a variety of gene sequences (qPCR master mix) for all organisms in the eDNA samples, including metabolic genes
- mRNA gene copy quantitation can be used to identify cyanobacterial toxin production genes and other genes encoding for proteins
- Note that RNA is ephemeral compared to DNA.
- Proteomics can determine what structural proteins are being produced

Cyanotoxins Analytical Methods

Detection Methods for Cyanotoxins

There is a diverse range of rapid screen tests and laboratory methods available to detect and identify cyanobacteria cells and cyanotoxins in water. These methods can vary greatly in their degree of sophistication and the information they provide. These methods include:

- Enzyme–linked immunosorbent assays (ELISA)
- Protein phosphatase inhibition assay (PPIA)
- Reversed-phase high performance liquid chromatographic methods (HPLC) combined with mass spectrometric (MS, MS/MS) or ultraviolet/photodiode array detectors (UV/PDA).
- Liquid chromatography/mass spectrometry (LC/MS)
- Conventional polymerase chain reaction (PCR), quantitative real–time PCR (qPCR) and microarrays/DNA chips

https://www.epa.gov/ground-water-and-drinking-water/detection-methods-cyanotoxins

The EPA Cyanotoxins Methods

EPA developed the following methods for the detection of cyanotoxins in drinking water and ambient freshwater:

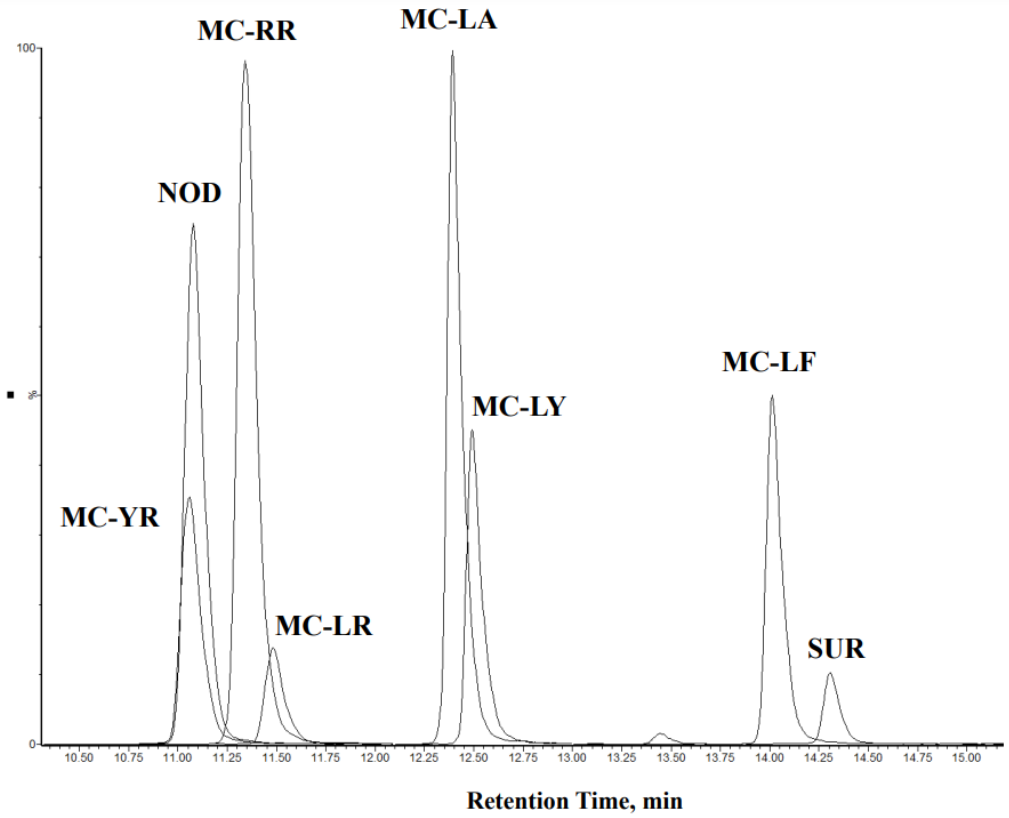
- Method 544: Determination of Microcystins and Nodularin in Drinking Water
- Method 545: Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water
- Method 546: Determination of Total Microcystins and Nodularins in Drinking and Ambient Waters
- Method for Determination of Cylindrospermopsin and Anatoxin-a in Ambient Freshwaters
- Method for Determination of Microcystins and Nodularin in Ambient Freshwaters

https://www.epa.gov/ground-water-and-drinking-water/detection-methods-cyanotoxins

EPA 544 (LC MS/MS for Microcystins and Nodularin) in Drinking Water

US EPA method 544, Determination of Microcystins and Nodularin in Drinking Water by Solid Phase Extraction and Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS), was developed to quantify selected microcystins and nodularins.

- It requires solid phase extraction sample preparation to achieve the sensitivity required to meet the microcystins advisory limits for six microcystin congeners.
- The method is designed to separately extract these cyanotoxins from intracellular and extracellular portions of the drinking water sample and combine the two extracts prior to analysis

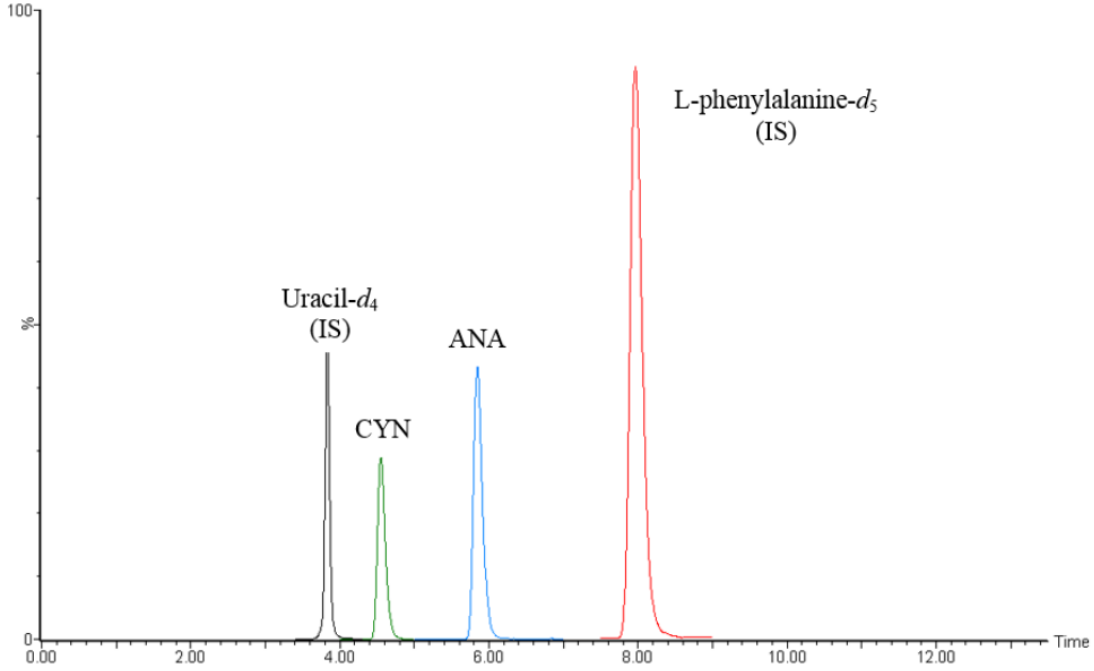


EPA 545 (LC MS/MS) – Anatoxin-a and Cylindrospermopsin

Determination of Cylindrospermopsin and Anatoxin-a in Drinking Water by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry (LC/ESI-MS/MS)

- Direct injection (no solid phase extraction required) of drinking water samples into the LC/MS/MS specifically for separation and detection of cylindrospermopsin and anatoxin-a. Given the strong signal to noise for these toxins, direct injection allows for rapid testing.
- By splitting samples and doing a filtered and unfiltered analysis, this method can determine the total or extracellular concentrations of these cyanotoxins in drinking water.
- Note – for ambient waters, there is the "Shoemaker Method" a single-lab method publication (EPA 2017): Ambient water anatoxins and cylindrospermopsin detection by LC MS/MS

Figure 1. Example Chromatogram of ESI (+) Transitions for Method 545 Analytes



EPA 546 – ADDA-ELISA, Microcystins (Total) in Ambient and Drinking Water

Determination of Total Microcystins and Nodularins in Drinking Water and Ambient Water by ADDA Enzyme-Linked Immunosorbent Assay

- ELISA kits sensitive to total microcystins use a variety of antibodies isolated against microcystin-LR and microcystin-RR congeners. They also use recombinant antibody fragments and antibodies against the amino acid ADDA, which is present in most of the congeners.
- These express a color signal, which are evaluated for intensity using a microplate reader at 450 nm to provide an estimated total microcystins concentration in µg/L.

A Note on ELISA Methods

Methods	Anatoxins	Cylindrospermopsins	Microcystins	Saxitoxins
Enzyme-Linked Immunosorbent Assays (ELISA)	Yes	Yes	Yes	Yes

Quantitative ELISA test kits are available for microcystins/nodularins (including ADDA-ELISA), saxitoxin, anatoxin-a, and cylindrospermopsin. Although they provide rapid results, ELISA kits generally have limitations in selectivity and are not congener-specific but require less expertise to run than LC MS/MS methods.

- For example, the ADDA-ELISA approach can detect over 100 congeners of microcystins

A Note on LC MS Methods

- LC MS approaches require the use of standards for quantitation
- At this time there are only standards available for a limited number of the known congeners of various cyanotoxins.

Methods	Anatoxins	Cylindrospermopsins	Microcystins	Saxitoxins
Liquid Chromatography Combined with Mass Spectrometry				
Liquid Chromatography Ion Trap Mass	Yes	Yes	Yes	Yes
Liquid Chromatography Time-of-Flight Mass Spectrometry (LC/TOF MS)	Yes	Yes	Yes	Yes
Liquid Chromatography Single Quadrupole Mass Spectrometry (LC/MS)	Yes	Yes	Yes	Yes
Liquid Chromatography Triple Quadrupole Mass Spectrometry (LC/MS/MS)	Yes	Yes	Yes	Yes

EPA, 2019

Comparative Matrix of Microcystins and Cylindrospermopsin Analytical Methods

Table 4: Analytical methods of Microcystin and Cylindrospermopsin detection and non-toxins in ambient water[1]

TEST/ METHOD	Analytical Target	APPROX. LIMIT OF QUANTIFICATION	LOCATION OF TEST	TIME TO RESULT	SCREENING ONLY TOOL
EPA Method 546 Adda-ELISA	Intracellular and Extracellular Microcystins	0.10–5.0 µg/L	Lab	~ 1 day	No
ELISA-DM Laboratory Test	Total Microcystins	0.010 µg/L	Lab	3 hours or less	No
ELISA Laboratory Test SAES (Abraxis)	Total Microcystin in marine/brackish water	0.016 µg/L	Lab	3 hours or less	No
ELISA Laboratory Test (Abraxis)	Total Cylindrospermopsin	0.05 – 2.0 µg/L	Lab	3 hours or less	No
ELISA Laboratory Test (Beacon)	Total Cylindrospermopsin	0.1-2.0 µg/L	Lab	~2 hours	No
HPLC-UV (PDA)	Total Microcystins-limited specificity	~0.3 µg/L	Lab	~ 1 day	No
Protein Phosphatase Inhibition Assay	Total Microcystins	0.02 µg/L	Lab	N/A	No
Microcystin Tube Kit (Abraxis)	Total Microcystins	0.1-5.0 µg/L	Lab	~45 minutes	No
Microcystin Tube Kit (Beacon)	Total Microcystins	0.3-5.0 µg/L	Lab	~1 hour	No
Abraxis Test Strip	Total Microcystins	1–10 µg/L	Field	~45 minutes	Yes
	Total Cylindrospermopsin	0.5–10 µg/L	Field	~45 minutes	Yes

From EPA's Recommendations for Cyanobacteria and Cyanotoxin Monitoring in Recreational Waters, 2019- EPA 823-R-19-001

Leduc et al. 2020
test strip multitoxin intercomparative with ELISA at a MN lake

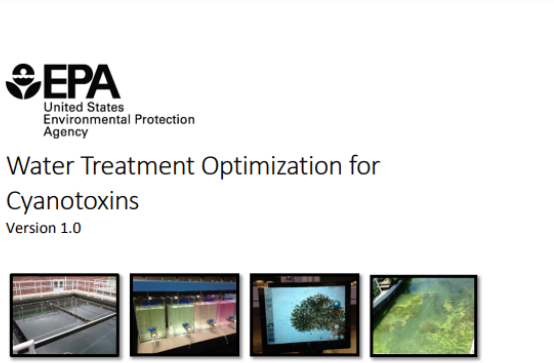
Sample Collection Considerations

Samples should be collected and held properly to ensure integrity. Some considerations:

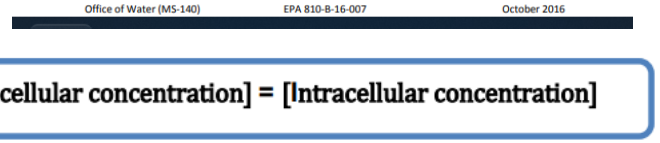
- *Collection* – Bottle type, volume, and preservative used depend on the laboratory and the method. Amber glass containers are typically recommended for the drinking water methods.
- *Quenching* – samples that include a residual disinfectant, e.g., chlorine, should be quenched immediately upon sampling. Sodium thiosulfate or ascorbic acid are typically used.
- *Chilling* – samples should be chilled immediately after collection, during shipping, and holding at the laboratory. Sample freezing may be appropriate to extend holding times, taking precautions to avoid breakage.

To Pre-Filter or Not to Pre-Filter? And What About Lysing?

- When preparing the sample for analysis, filtering may help separate intracellular from extracellular toxins, if needed.
- The ELISA and LC/MS/MS methods typically require lysing the cyanobacterial cell walls in the samples prior to analysis in order to determine total toxins. The most common lysis approach is to freeze and thaw the entire water sample three times prior to analysis.
- Samples post lysis can be examined microscopically if there are concerns about whether samples are fully lysed.



treatment approaches can differ significantly based on whether or not the cyanotoxins are primarily intracellular or extracellular (located outside the cyanobacteria cell)



https://www.epa.gov/sites/default/files/2018-11/documents/water_treatment_optimization_for_cyanotoxins.pdf

Methods Comparison Matrix

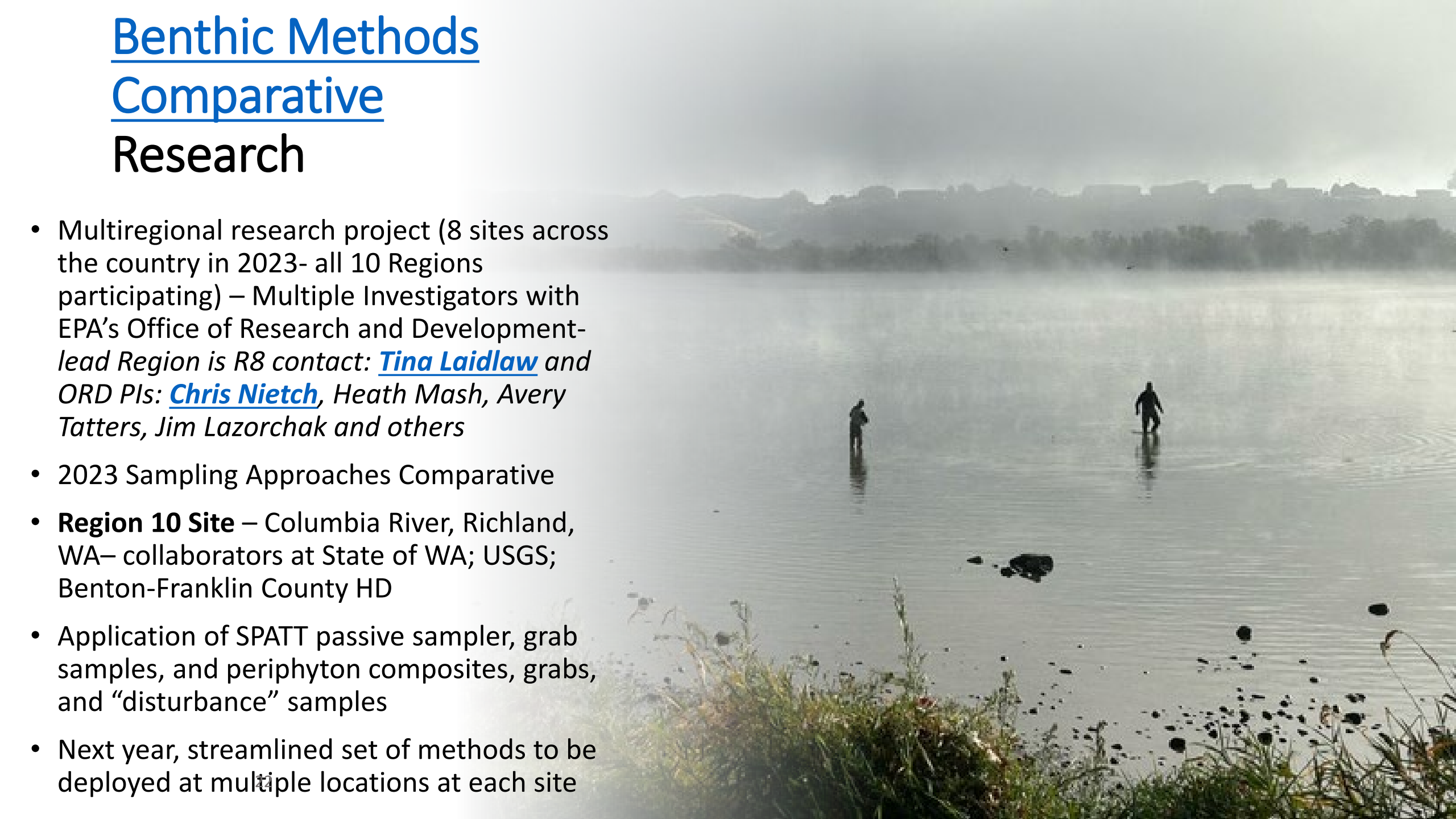
Toxin and method	Bottle Type	Holding Time and Temperature	Lysis step	Filtration required ?	Multi-or single sample runs?	Typical MRL (lab/instrument-dependent)	Preservative and/or quenching agent?
USEPA 544 6 MC Congeners + Nod	Amber glass with teflon-lined screw caps	Chill (10°C) upon collection and transport. Samples stored in the lab must be held at or below 6°C until extraction, but should not be frozen. Water samples should be extracted as soon as possible after collection but must be extracted within 28 days of collection. Extracts must be stored at ≤ -4°C and analyzed within 28 days after extraction.	Sample is filtered. The filter is placed in a solution of methanol containing 20% reagent water and held for at least one hour at -20 ºC to release the intracellular toxins from cyanobacteria cells captured on the filter. The liquid is drawn off the filter and added back to the aqueous filtrate.	Y (filtrate separated from filter and processed separately, then recombined before analysis)	Single but with an autosampler can run multiple samples per day	LCMRLs in reagent water 0.0029 – 0.022 µg/L depending on congener	Y – check method
USEPA 545 Atx/Cyl	Amber glass with teflon-lined screw caps	Chill (10°C) upon collection and transport to lab (first 48 hours). In the laboratory, samples must be stored at or below 6°C and protected from light until analysis. Samples must not be frozen. Samples should be analyzed as soon as possible, but must be analyzed within 28 days	A triple freeze and thaw process may be used on samples prior to filtration and analysis in order to address the potential presence of intact algal cells in finished water samples.	Y- post lysis (see method)	Single but with an autosampler can run multiple samples per day	LC MRLs in reagent water were 0.018 and 0.063 µg/L for anatoxin-a and cylindrospermopsin respectively	Y – check method
USEPA 546 Total MC/Nod	Amber glass with teflon-lined screw caps	Chill (10°C) upon collection and transport. Freeze samples upon arrival at the laboratory. Must be analyzed within 14 days of collection.	Mix thoroughly and immediately transfer 5 to 10 mL of each field sample into a 40 mL vial to begin three freeze-thaw cycles.	Y post lysis (see method)	Multi-well plates (96); 3-4 hours per runtime. One plate per kit.	Nominal MRL 0.30 µg/L (see method)	Y- check method

Methods Development for Fish Tissue-Known Status

FDA has developed saxitoxins methods for fish and shellfish tissue

- FDA is developing cyanotoxins methods for LC-MS/MS for analyzing shellfish tissues. Currently they have single-lab validated a method for measurement of MC-LA, -LF, -LR, -LW, -LY, -RR, -YR, and nodularin-R in clams, mussels, and oysters with a publication in development.
- EPA ORD Development of methods for measuring total microcystins in Fish Tissue using the 2-methoxy-3-methyl-4-phenylbutyric acid (MMPB) procedure – broader confirmation method. Publication in development.
- Many studies have used ELISA and LC MS/MS to analyze cyanotoxins in fish tissue. For example, Hardy et al. 2015 investigated cyanotoxins accumulations in freshwater fish tissue in Washington State using ELISA and LC MS/MS. They found that microcystins or microcystin-like compounds were elevated in fish liver relative to muscle and other tissues (liver>gut>muscle).
- EPA 2022 Occurrence and Effects of Cyanotoxins in Fish and Shellfish Webinar Presentations

Benthic Methods Comparative Research



- Multiregional research project (8 sites across the country in 2023- all 10 Regions participating) – Multiple Investigators with EPA's Office of Research and Development- *lead Region is R8 contact:* ***Tina Laidlaw*** *and ORD PIs:* ***Chris Nietch****, Heath Mash, Avery Tatters, Jim Lazorchak and others*
- 2023 Sampling Approaches Comparative
- **Region 10 Site** – Columbia River, Richland, WA– collaborators at State of WA; USGS; Benton-Franklin County HD
- Application of SPATT passive sampler, grab samples, and periphyton composites, grabs, and "disturbance" samples
- Next year, streamlined set of methods to be deployed at multiple locations at each site

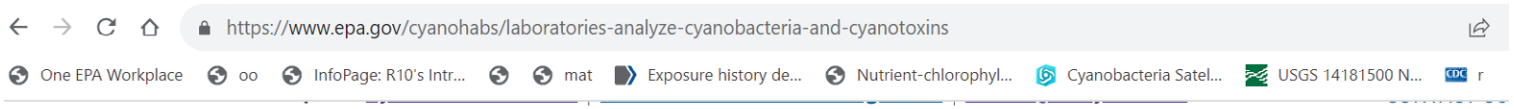
View Through the Bathyscope

- 100% coverage?

Collecting a Known Disturbance Volume or Individual Grabs

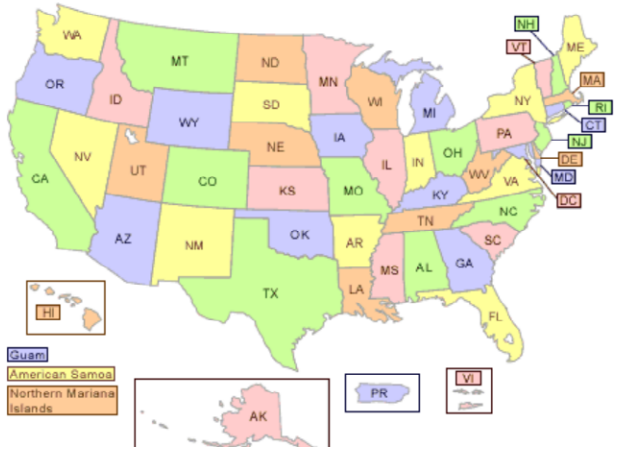
- Benthics are challenging to assess – can be heterogeneous in space and time
- Comparing periphyton “picks” to disturbance of a known area and volumetric capture

Laboratories Lists

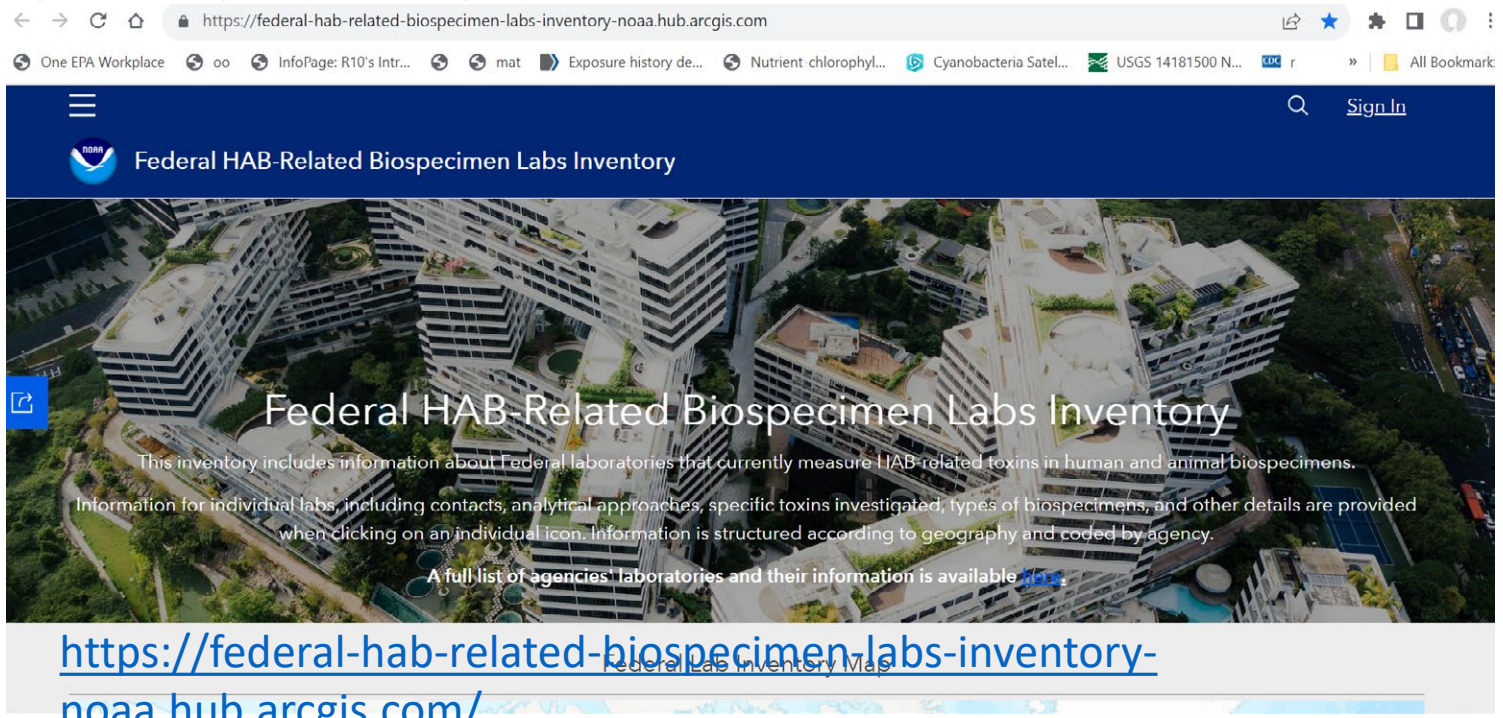


Laboratories that Analyze for Cyanobacteria and Cyanotoxins

States and territories that have laboratories available to analyze water samples for cyanobacteria and cyanotoxin can be found by selecting the state on the map.



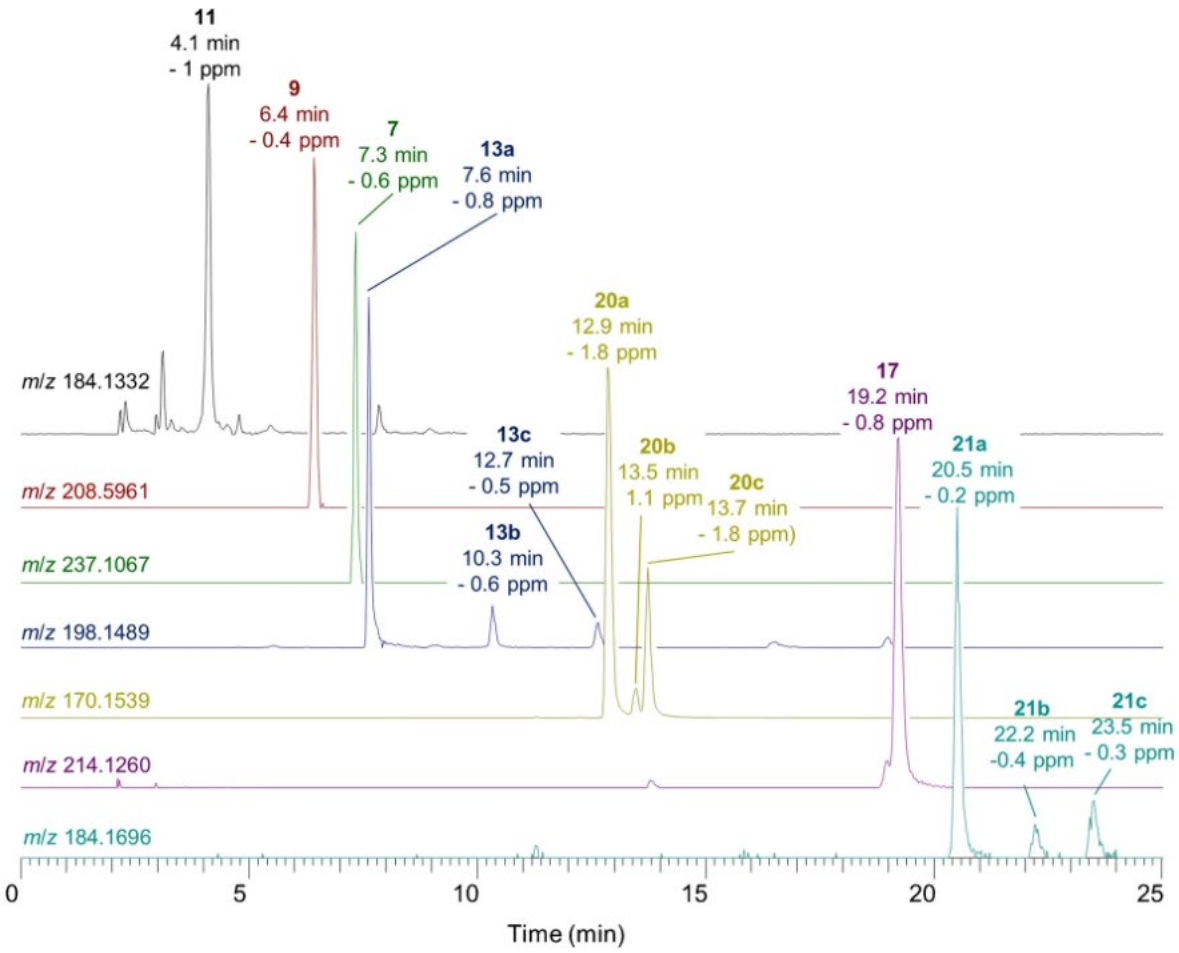
https://www.epa.gov/cyanohabs/laboratories-analyze-cyanobacteria-and-cyanotoxins



https://federal-hab-related-biospecimen-labs-inventory-noaa.hub.arcgis.com/

Future Methods Development Needs

- More standards for more cyanotoxins
- Broader application of high-resolution mass spec
- Can we use quantum yield of fluorescence or gene expression to determine state of benthics/lifestage?
- Metabolomics expression geography
- In situ eDNA and eRNA collection
- Core sediment studies for quiescent waters
- Deploying SPATTs/passive samplers on AUVs

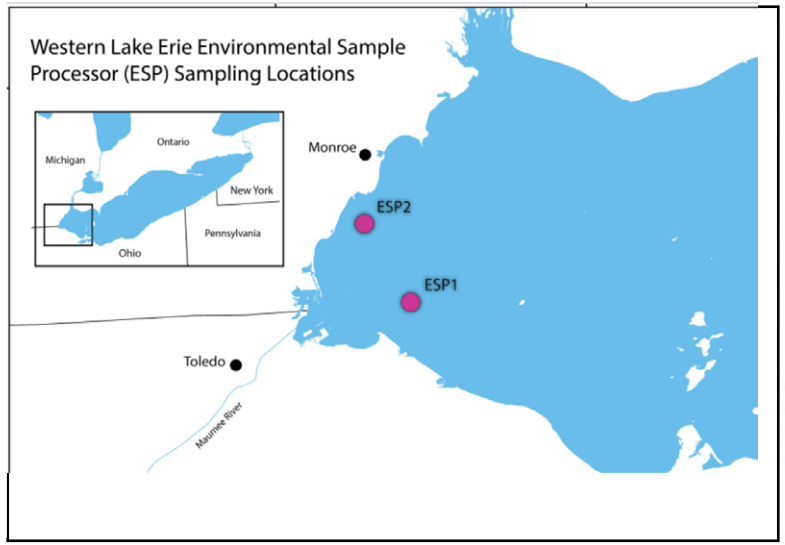


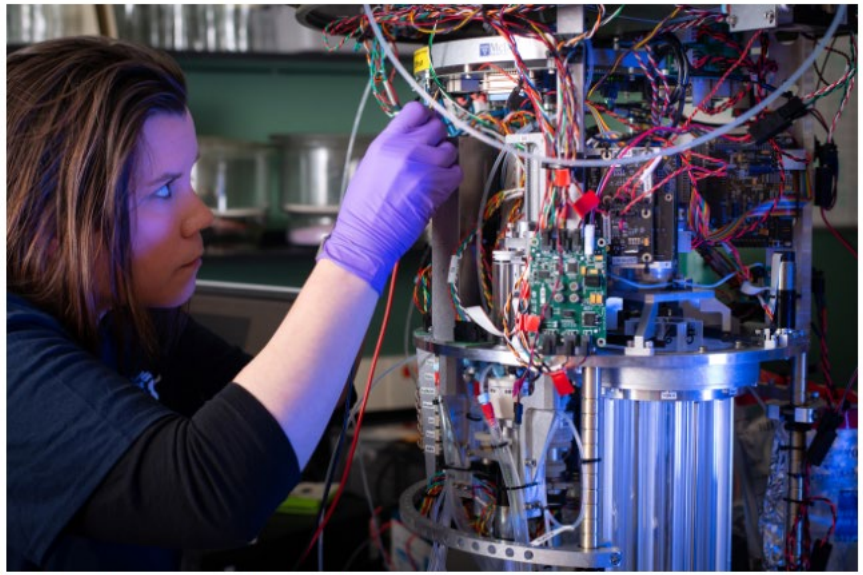
LC-HRMS of anatoxin congeners and derivates from [Beach et al. 2023]

Future Developments: Can We Bring the Lab to the Field?

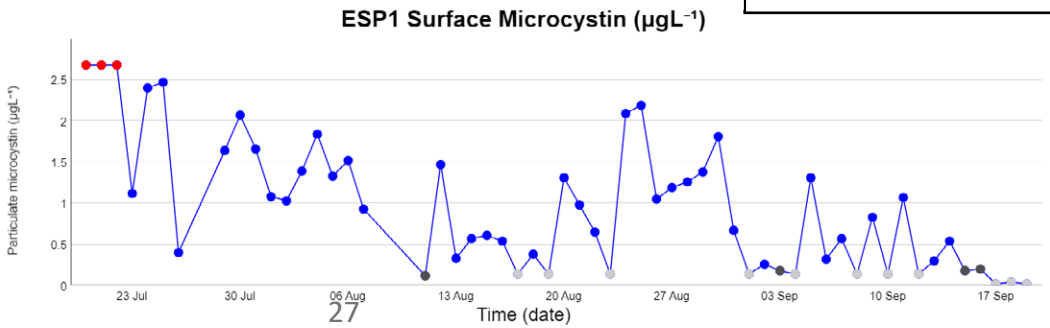
- Can “lab in a can” or robotic sampler technology become more affordable and appropriate for all fresh waters?

  eDNA and toxins semi-continuously!





NOAA GLERL research associate Danna Palladino prepares an Environmental Sample Processor (ESP) for deployment. NOAA GLERL researchers use a variety of advanced technologies to study and monitor harmful algal blooms (HABs). This allows NOAA to provide drinking water managers with data on harmful-algal toxicity in near real-time, before the water reaches municipal water intakes. This data, coupled with the NOAA Lake Erie HAB Forecast provide water managers with more precise bloom location, projected direction, intensity, and toxicity. Photo Credit: Dave Brenner, University of Michigan.



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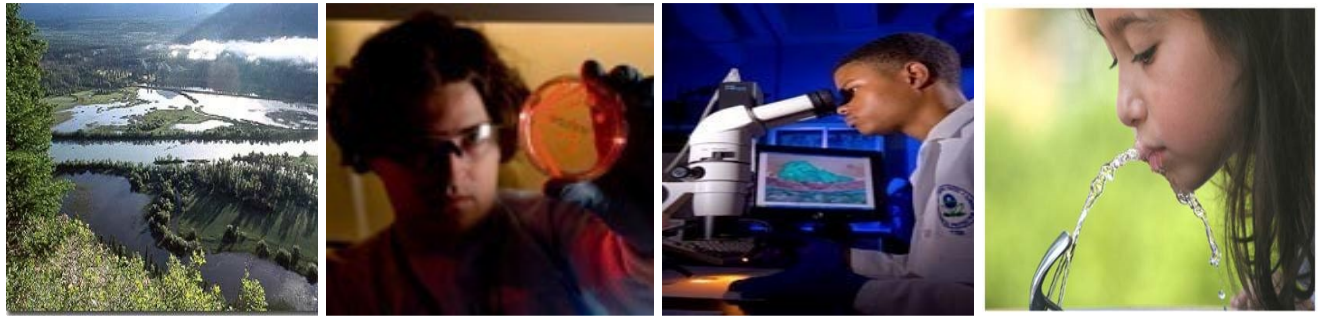
Algal Toxins Analysis Capabilities at the Region 10 Laboratory

October 18, 2023



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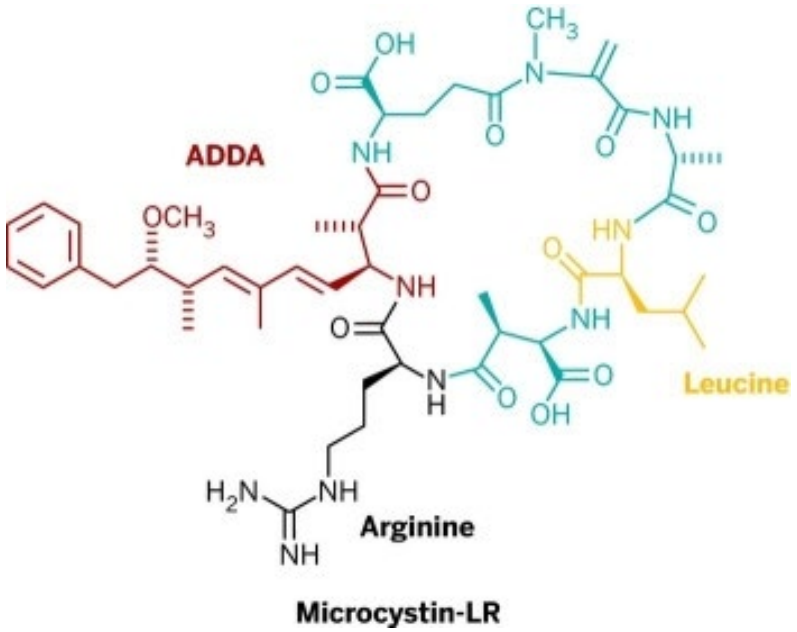
EPA HAB Methods

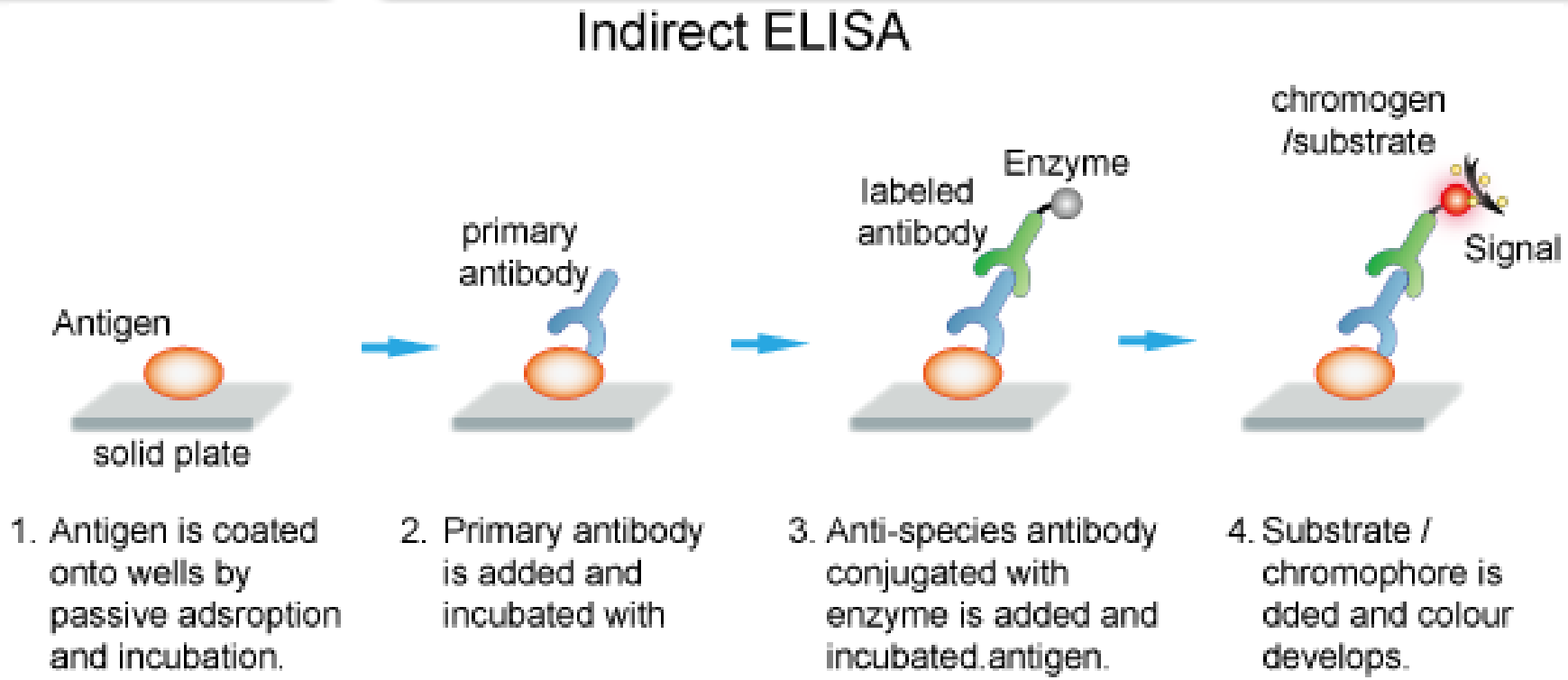
- Under UCMR 4, EPA has published 3 methods for HABs
  - Method 546 is an ELISA-based method for “total microcystins”
  - Method 545 is a LC/MS/MS method for Cylindrospermopsin and Anatoxin-a
  - Method 544 is an LC/MS/MS method for 6 microcystins and Nodularin
  - Method 545 was also applied to surface waters (EPA publication)

Method 546 for Total Microcystins

- The number of microcystin congeners is believed to be over 100
- The Enzyme –Linked Immunosorbent assay (ELISA) antibody-based method was developed for microcystins that contain the ADDA subunit, which is common



Method 546



- Enzyme-linked Immunosorbent Assay Method uses a sheep polyclonal Ab
- Anti-sheep HPP-conjugate turns over substrate to produce colored product. Timing is critical

Method 546 Calibration

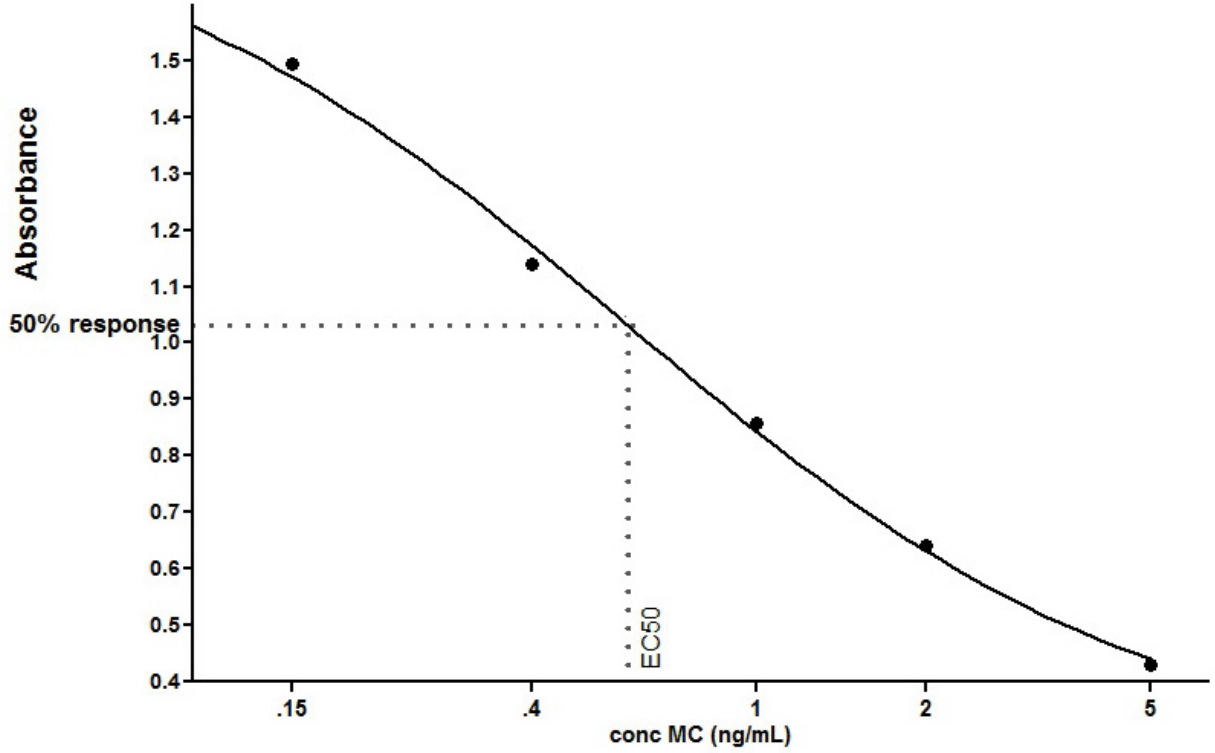
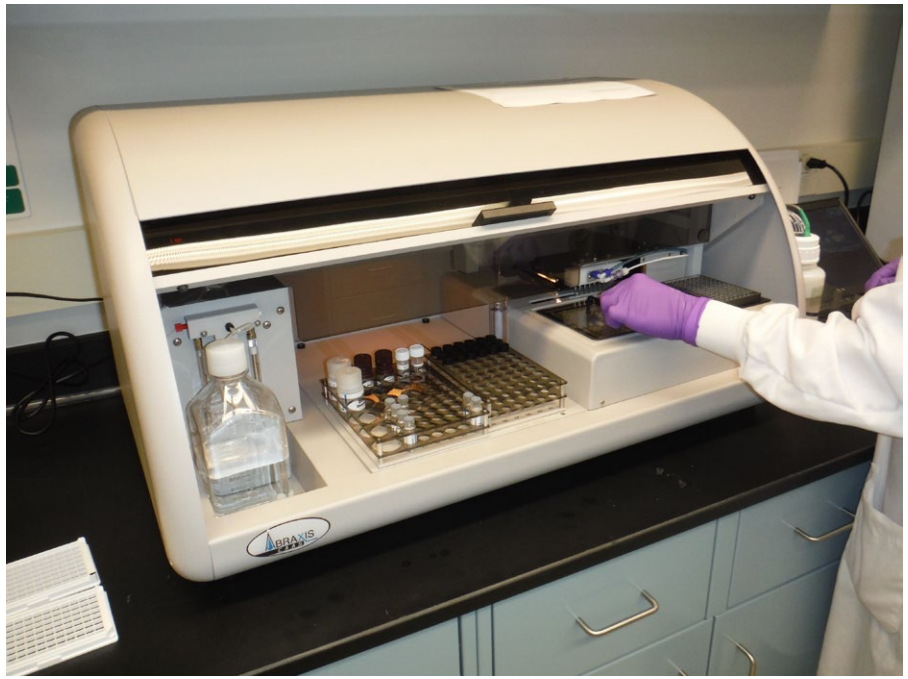


Figure 1. ELISA calibration curve with calculated EC_{50}. Note: x-axis uses a logarithmic scale.

- The x-axis is logarithmic; absorbance decreases with increasing concentration
- Method Reporting Limit around 0.3 µg/L (same as the HA level for young children)

Method 546 Instrument

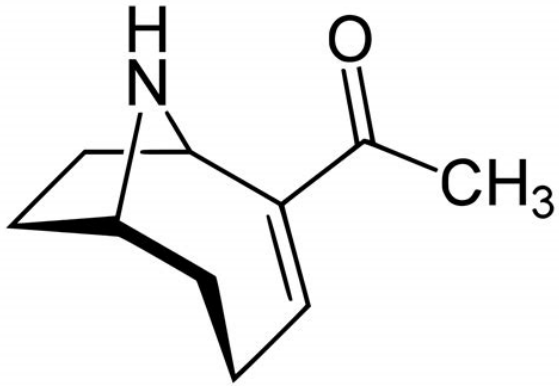


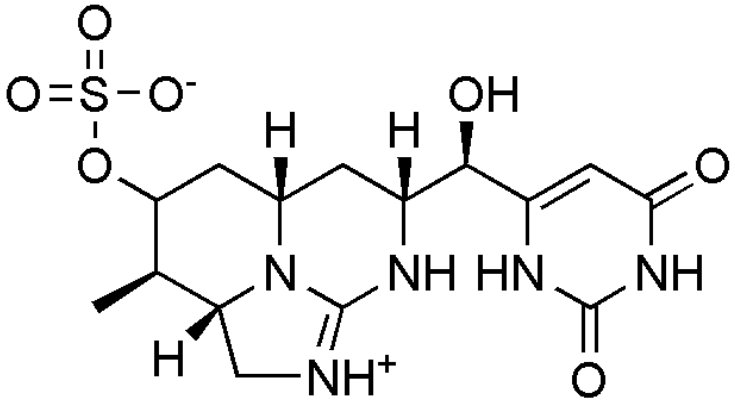
- Making decisions at the MRL requires careful control of method, but even then, the QC criteria is +/- 50%
- Still, this method is most protective of human health

Method 545 Instrument

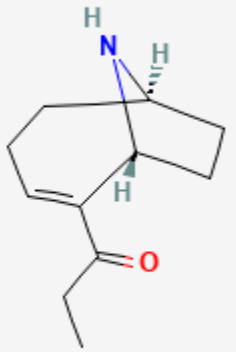
Method 545 for Cylindro and Anatoxin-a

- Anatoxin-a (top) and Cylindrospermopsin (bottom) are analyzed in a single drinking water method

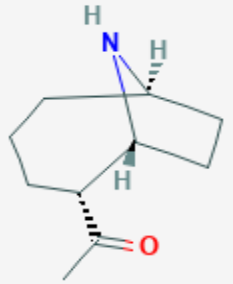




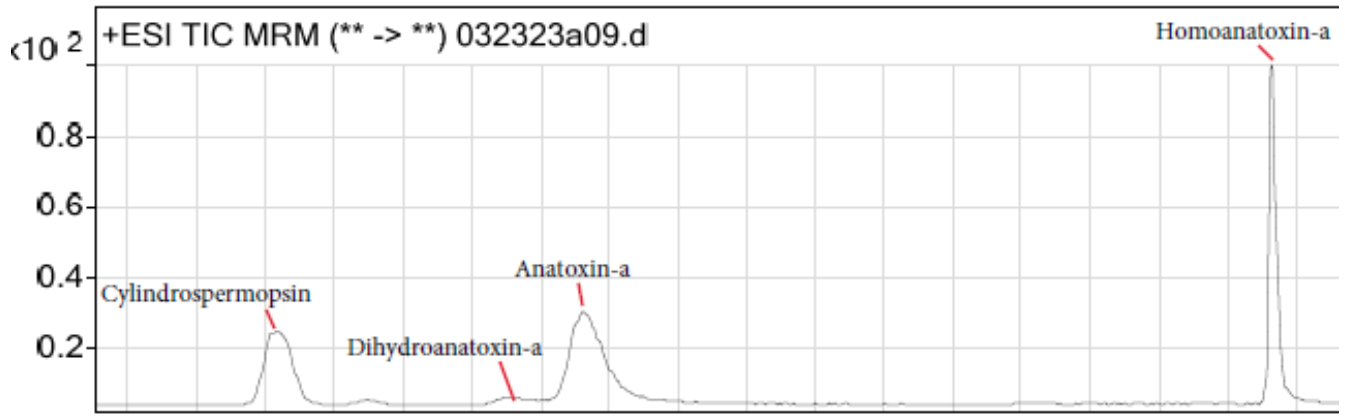
Homoanatoxin-a



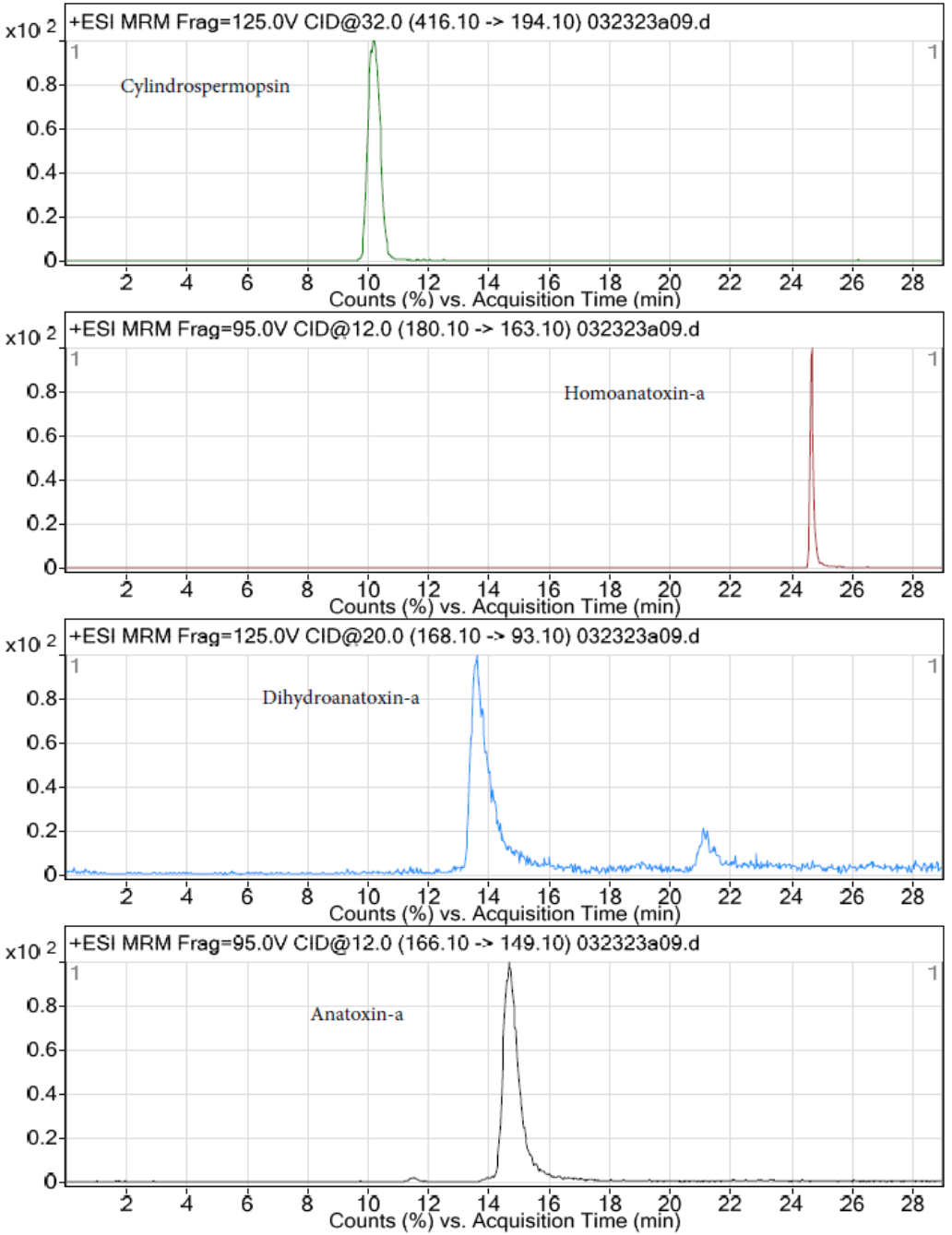
Dihydroanatoxin-a



Method 545



- Chromatogram with new internal standards:
  - Cylindrospermopsin-N15, and
  - Anatoxin-C13


+ESI MRM Frag=125.0V CID@32.0 (416.10 -> 194.10) 032323a09.d
Cylindrospermopsin
+ESI MRM Frag=95.0V CID@12.0 (180.10 -> 163.10) 032323a09.d
Homoanatoxin-a
+ESI MRM Frag=125.0V CID@20.0 (168.10 -> 93.10) 032323a09.d
Dihydroanatoxin-a
+ESI MRM Frag=95.0V CID@12.0 (166.10 -> 149.10) 032323a09.d
Anatoxin-a
x10 2
Counts (%) vs. Acquisition Time (min)

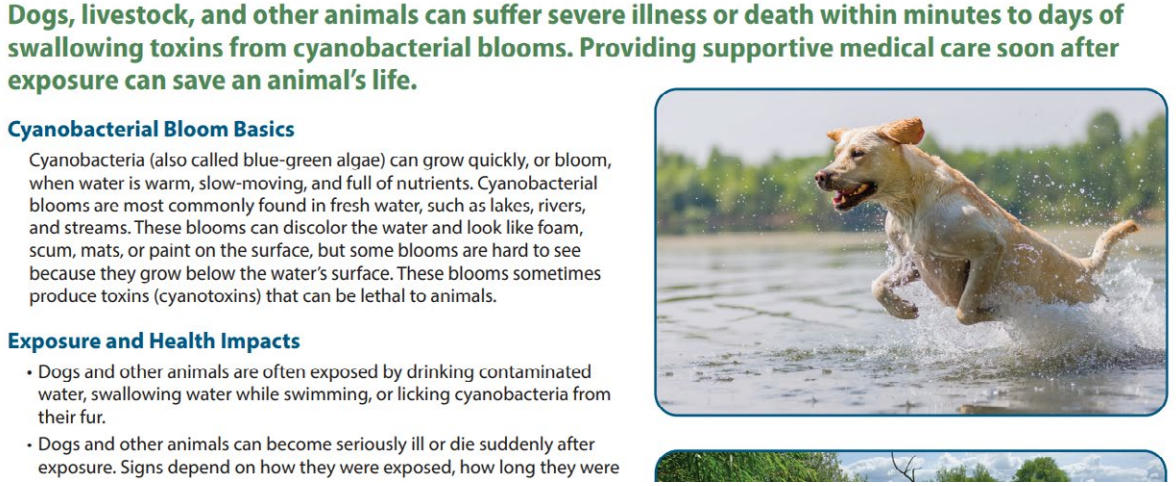
Summary of R10 Lab Capabilities

- Method 546 Reporting Limit for Total Microcystins and Nodularins: 0.30 µg/L

- Method 545 Reporting Limits: Anatoxin-a 0.051 ug/L, Cylindrospermopsin 0.11 µg/L, Dihydroanatoxin-a 0.20 µg/L, Homoanatoxin-a 0.050 µg/L

Columbia River Tri-Cities Support, 2021

- 4 dog deaths and several illnesses after recreation on Columbia in vicinity of 6 drinking water facilities (~250K served)
- Ambient cyanotoxins detected from Sept-Nov
- Finished water breakthrough of anatoxin-a, but below the state (WA) health guidance value (0.3 μg/L) for ~6 weeks
- EPA R10 provided weekly analytical support (anatoxin-a by LCMSMS, 24-hr TAT) and technical support for the duration of the event
- Benthic HABs identified as a potential issue



Dogs, livestock, and other animals can suffer severe illness or death within minutes to days of swallowing toxins from cyanobacterial blooms. Providing supportive medical care soon after exposure can save an animal's life.

Cyanobacterial Bloom Basics

Cyanobacteria (also called blue-green algae) can grow quickly, or bloom, when water is warm, slow-moving, and full of nutrients. Cyanobacterial blooms are most commonly found in fresh water, such as lakes, rivers, and streams. These blooms can discolor the water and look like foam, scum, mats, or paint on the surface, but some blooms are hard to see because they grow below the water's surface. These blooms sometimes produce toxins (cyanotoxins) that can be lethal to animals.

Exposure and Health Impacts

- Dogs and other animals are often exposed by drinking contaminated water, swallowing water while swimming, or licking cyanobacteria from their fur.
- Dogs and other animals can become seriously ill or die suddenly after exposure. Signs depend on how they were exposed, how long they were

For more information on animal exposures and health effects, go to CDC's website Veterinarian Reference Card

Other Developments

* Collaborated with Oregon DEQ to test the Streptavidin Enhanced Sensitivity (SAES) assay kit which can result in an MRL one third of the Method 546 value, 0.3 μg/L (EPA Health Advisory Level for small children). This provides a small but critical window for early warning. Publication soon.

* Total anatoxin ELISA kit also being tested.

Additional Resources List

- EPA Main Cyanotoxins Methods Page: https://www.epa.gov/ground-water-and-drinking-water/detection-methods-cyanotoxins
- EPA Monitoring and Responding to Cyanotoxins in Recreational Waters Guidance (2019)
- APHL Guidance 2021- Cyanotoxins: A Guidance for Public Health Laboratories
- ITRC HCB-1 and HCB-2
- Toxic Cyanobacteria in Water – 2nd Addition: WHO 2021
- Canadian Guidance on Cyanobacteria in Freshwater – NCCEH 2023: https://ncceh.ca/resources/subject-guides/cyanobacteria-freshwater

Contacts

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EPA Region 10 (Pacific Northwest)

Serving Alaska, Idaho, Oregon, Washington, and 271 Tribal Nations

Sawtooth Mountains near Stanley, Idaho.