Lab Analysis & Testing Methods from

Information Management & Quality Assurance Center

State Water Resources Control Board
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Overview

- Focus on lab analysis of cyanobacteria and it’s toxins
- How to determine if water samples contains
  - Toxins
  - Toxin producing cells
  - Cyanobacteria cells
Many genera of cyanobacteria have the capability of producing toxins – but not all. If cyanobacteria produces toxins – cyanobacteria contain toxins within intact cells. Then internal toxins are released to the surrounding environment when cells are damaged or die. [by natural processes (e.g. cell death, predation) and artificial processes (e.g. algaecides, laboratory processes]

Once toxins are released into the surrounding water, the toxins can persist for weeks to months. Some classes of toxins are more stable than others.

The timing of toxin release from cells has an exception: Research indicates that cyanobacteria cells capable of producing cylindrospermopsin can release toxins while living, about half the toxins can be found in the surrounding environment (US EPA, 2015)
Cyanobacteria Cell & It’s Toxins

- Laboratory process used to release toxins from cells – called “lysis”
- Cell lysis is required to measure “total toxin” concentration
  - Common processes: multiple freeze/thaw cycles, sonication, chemicals, others

- The measurement of “total toxin” requires the process of cell lysis
- The lab process of cell lysis is done to break open all cells
- There are several different procedures for cell lysis – each successfully disrupts varying % of cells
- This image shows the lysis process of sequential “freeze-thaw” cycles
- Other cell lysis processes include: sonication, freeze drying, and chemicals
What is meant by collecting a water sample for cyanotoxin lab analysis? Includes samples that capture planktonic blooms and benthic blooms. Water sample includes a combination of water and particles (scum, algal mats, visible colonies). Water sample can also include collection of benthic algal mats that are either attached/detached. Toxins are not visible and toxin presence cannot be determined by microscopy. Detection of toxins is done by collecting samples and conducting analysis of the cyanobacteria and the toxin levels. In flowing water, samples should be focused along shorelines where recreational users and animals are entering the water, backwater areas where algae and toxins may accumulate, and upstream and downstream of the area of concern.

Images:
Upper left-Regional Water Quality Control Board, 2015
Lower left-USGS, J. Graham
Middle-USGS, 2008
Upper right- unknown
Lower right-New Zealand, Ministry for Environment
HAB Sampling

- How are water samples tested by lab procedures?

  Depends on the question being asked, directs which “fraction” to measure:
  - What is the amt. of extracellular toxins (i.e. test the dissolved fraction or filtrate) \textit{OR}
  - What is the amt. of intracellular toxins (i.e. test the particulates – colonies, mats, scum) \textit{OR}
  - What is the amt. of toxins in the bloom sample, measure the “\textbf{total}” toxins (i.e. both fractions)

- Communication with the lab is critical to direct them to measure the fraction that will inform your monitoring question
Phases of toxin – each can be measured by lab methods – important to understand when requesting lab analysis

(image: **Total toxin** = extracellular (dissolved) + intracellular (particulate)

Total toxin concentration – Measurement of “total toxin” (both intra + extracellular toxin) estimates the worst case exposure. This is because the intact cells are broken open to release the toxins into the sample, this measures the potential toxin concentration if all the cells release toxins into the environment. Researchers have measured fluctuating bloom abundance (total cells) and toxin concentration within 12-24hrs.

Intracellular (particulate phase) – toxins located within the cell particles and absorbed to other materials. The lab process of lysing breaks open the cells to release the toxin into liquid.

Extracellular (dissolved phase) – toxins previously released by cells and dissolved in water.

- The two phases are separated by vacuum filtration
- Benthic algal mat (scrapings or detached) follow particulate phase testing.

For more info on testing benthic cyanobacteria in CA refer to Fetscher et al. 2015
- Cyanotoxins commonly found in CA, include toxins from 2 groups – hepatotoxins (impacts the liver) and neurotoxins (impacts the neurological system).
- The compounds MC and nodularins contain the “ADDA” side group (circled in green). This ADDA group is important when considering ELISA methods.
- Microcystins are the most commonly detected cyanotoxin in CA according to publically available monitoring reports.

- It’s important to know what cyanobacteria is in the bloom material, this is done by identifying the genera/species or toxin genes.
- Some species can produce more than 1 toxin.
- Toxin producing strains are not created equal!
  - Strains produce different toxins & variants
  - Strains produce toxins at different rates and amounts
Microcystins—Special Considerations

- 100+ variants discovered
  \cite{Walker2008, US-EPA2015}
- Variants exhibit different toxicity \cite{Primoz2013}

- Calibration standards available for \textasciitilde{}12 variants
- Identification of variants – useful to characterize the risk of the toxins produce by bloom cycles
- Measured by both types of analytical methods:
  \begin{itemize}
    \item ELISA results in quantification of \textit{total microcystins} (MC)
    \item LC-MS results in quantification of \textit{individual} MC variants
  \end{itemize}

- Microcystins are unique and requires special considerations prior to requesting lab analysis and data interpretation
- Microcystins are a \textit{class} of cyanotoxins synthesized by some cyanobacteria
- Approximately 100+ variants of the microcystin compound have been discovered and are abbreviated by “MC”. Microcystins consist of similar chemical structure, they are differentiated by the molecules that are substituted in 2 locations on the compound – these locations are depicted by the “X and Y” in the image. These different X and Y groups distinguish MC variants and are commonly named: MC-LR, MC-LA, MC-RR, MC-YR, etc.
- The toxicity of the variants are not the same and can vary by several magnitudes. For this reason it is important to identify which variants are produced by a bloom.
- Current laboratory analysis capabilities are limited to testing a portion of the discovered variants (~12 variants). As analytical standards are developed more variants can be identified by analytical methods.
- Both ELISA and LC-MS methods can detect these variants; however, each method measures something different (group of MCs vs. individual MC variants). Data from both of these analytical methods can not be directly compared. To use data for observing trends, its recommended to utilize a single method over time so that the data can be compared.
- Communication with the laboratory is recommended to learn about their
available testing methods.
-Visual observation cannot determine the presence of toxins
-There are many types of analytical methods to measure these toxins; however, the methods differ by what they measure, accuracy, precision, cost, detection limits, and availability.
-Table presents 3 commonly available analytical methods
-No one way of measuring cyanotoxins is best – analytical methods continue to advance

-Sensitivity definition from US EPA: The capability of a method or instrument to discriminate the parameter of interest at the level of interest. Terms sometimes used to describe sensitivity include Method Detection limit (MDL), Limit of Detection (LOD), and Limit of Quantitation (LOQ) (US EPA, 2012)

-Selectivity definition from US EPA: Selectivity refers to the ability to correctly identify the analyte(s) of interest in the presence of expected chemical/physical interferences. (US EPA, 2005)

-ELISA method – can be designed to result in qualitative, quantitative, or semi-quantitative data. Quantitative data is obtained when the assay response is compared to a standard curve (prepared from measuring 5+ calibration standards)

-Other analytical chemistry instruments can be utilized for quantification of cyanotoxins
(e.g. HPLC, LC/TOF MS). For more information, refer to US EPA HABS webpage and USGS recent presentation at http://ks.water.usgs.gov/static_pages/studies/water_quality/cyanobacteria/loftin-analytical-Methods.pdf
What is it: rapid antibody based biological method
-The assay involves biological reaction of antigen binding to specific antibodies -> reaction produces a response (i.e. color change) that is measured

Images: Left side-www.StateofFlorida.habs.gov; Middle-Wikipedia; right side- www.ucf.edu
ELISA methods are available for a limited groups of cyanotoxins – currently each kit detects a single class of toxins per run. Check with your labs for ELISA availability since commercial capabilities are rapidly changing (ELISA tests for microcystins, nodularin, and anatoxin-a are most commonly available).

What does it measure? The assay is designed to react to a particular fragment/chemical group on the toxin compounds, so any toxin compound with that fragment produces a positive reaction. The reaction is depicted by a color change.

-Microcystins test kits are designed specific to MC-LR (single MC variant) or the ADDA subgroup found on the microcystins compound (i.e. the kits will measure a different portion of all MCs). To learn what the commercial kit is designed to react to, refer to the manufacturer and/or lab. It is useful to learn which kit or kits the lab will use for analysis, this info is useful when interpreting the data and comparing data obtained from multiple labs. (Samdal et al. 2014)

What are the results? Measures total toxin in ppb range

-Microcystins test reports total Microcystins concentration

-Kits report different cross reactivities (amount of reaction) to microcystin variants. Broad cross reactivity improves detection to the broad class of microcystins.

-To learn how strongly the test reacts to specific microcystin variants, refer to the
kit’s cross reactivity provided by the kit manufacturer and/or lab.
-Cross reactivity: response to a single variant is not the same to another variant meaning that the method doesn’t react as strongly to each variant.

Limitations: matrix interference found in complex samples, false positive results, variable cross reactivities (Merel, et al. 2013; Triantis, et al. 2010)
-Recommend running confirmation test on positive ELISA results to address concerns of false positives.
-The occurrence of false negatives is lower and recommended to confirm a smaller percentage of negative ELISA results.

Examples of its use: quick screening of samples, large batch processing
How to Measure Toxins?

LC-MS and LC-MS/MS Methods

What is it? Uses high tech instruments to conduct chromatography and mass spectroscopy
- analytical chemistry method
- analyzes toxins with greater specificity and selectivity

Images: left side-www.Umass.edu; right side-Agilent Technologies, Toni Hall, UK.
Monitoring for Microcystins in Raw Water Supply Reservoirs using the Agilent LC/MS
Or www.thermofisher.com
What does it measure? The response from the instruments, within an expected range of your targets, is compared to a standard curve to estimate the concentration of the individual compounds.

What are the results? Quantifies the concentration of individual compounds based on known reference standards. For microcystins analysis, note that the method measures individual MC variants and not the sum of MCs like with ELISA methods. To sum MC results, it's recommended to analyze 4+ MC variants and sum the individual concentrations. However, when reporting results identify which method was used since ELISA and LC-MS results cannot be directly compared.

Limitations-the methods can identify and measure toxins with available reference standards. Communicate with the lab to learn which toxins and MC variants are available.

Examples of its use-
-Method has low quantification limits (measures low concentrations of toxins) which is useful when comparing to low advisory thresholds.
-Often used as a second confirmation test when screening methods (such as ELISA) are utilized in a monitoring program.
-Useful when characterizing microcystin toxins from a bloom by identifying which
MC variants are being produced. This informs risk assessments since MC variants exhibit variable toxicity.
How to Detect Potential Toxin Production?

By PCR/qPCR methods

*What is it?* Utilizes Polymerase Chain Reaction (PCR), quick molecular biology method

*What does it measure?* Detects toxin producing genes within bacterial DNA material

*What are the results?* Reports number of gene copies per volume of sample (copies/mL)

- Greater number of gene copies, greater capability of toxin production within bloom
- Can correlate gene copies to toxin concentration & cell counts

-PCR method determines presence of toxin genes, *qPCR method measures the quantity of toxin genes*
- *qPCR* Method measures genes associated with production of MC, anatoxin-a, cylindrospermopsin, and saxitoxin
- Positive detections indicates that the gene is available in the bloom material and may produce toxins (the test doesn’t indicate that the cells are actively producing the toxin)
- Negative detections indicates that the gene for a particular toxin is not in the bloom material (captured by the sample) and likely not produced. Cyanobacteria species can produce more than 1 cyanotoxin

- *qPCR* results can be correlated to potential toxin concentration and cell counts, contact lab researchers to incorporate qPCR screening into monitoring design.
- The number of toxin gene copies per cell can be variable, researches report 2-10 copies of gene per cell. So 1 copy of the microcystin gene is not equivalent to 1 cell count.

For example, *Microcysistis* genera known to have 2-10 genome copies per cell (Otten, T. interview). Lab researchers often assists with data analysis to interpret correlation of # gene copies/mL to # cells/mL.

- Researchers Otten & Paerl, created a comparison of *qPCR* results vs. cell counts:
when toxin genes exceed 5,000 gene copies/mL its equivalent to cell counts of 40,000 cells/mL (Otten & Paerl, In press)

- If interested in detection/measurement of taste & odor compounds, the qPCR method can be used to detect the genes responsible for production of these nuisance compounds instead of toxin producing genes
Examples of regional use of qPCR methods:
- Klamath Basin studies ongoing since 2007
- SF Estuary studies since 2011

Procedure:
- About 3 hour process
- Sample (water sample or algal mat/particles on filter)
- Extract DNA
- PCR platform (assay run in individual tubes or up to 96-well microtiter plates)
  - Large batch processing available by conducting PCR with 96-well plates
- Add premixed reagents (primers, probe, etc.)
- Run on instrument (Automated amplification of target via thermocycler instrument)
- Analyze results with PCR analytical software -> data used for gene detection, quantification, genotyping, others uses
Forecasting toxic blooms:
- forecasting is done by determining if the bloom has the capability of producing toxins. For example, if the bloom material results indicate high # of cyanobacteria cells and increasing amount of genes for anatoxin-a production its reasonable to predict that the increasing bloom may produce anatoxin-a.

Informs toxin testing based on detected genes:
- Note that some genera/species can produce more than 1 toxin type.
- If a particular class of toxin genes are found in the bloom material, then toxin analysis can be conducted on a smaller set of toxins.
- reduces costs of toxin analysis since the results direct which toxins should be tested for a particular bloom.

Correlate toxin concentration to # of toxin gene copies: (Refer to Otten, T. et al. 2012. or contact the Klamath Basin Monitoring Program for more info)
- Since advisory thresholds/action thresholds are based on toxin concentration or cell counts, researchers are correlating these parameters to # toxin gene copies. This is a useful quick tool to guide what toxin testing is needed or forecast potential toxic blooms.

Characterize cyanobacteria blooms, informs risk assessments:
- What is the total amount of cyanobacteria cells and is it increasing/decreasing?
- What genera/strains make up the bloom?
- What toxins can the bloom produce?

Locate source of toxic cyanobacteria cells: Refer to Otten, T. et al. 2015 for Klamath Basin study

Note: when cells decay and DNA is released, DNA is unstable in natural environment. It's important to collect intact cells to measure the DNA. However, toxins can be stable in the environment for weeks to months.
How to Measure Quantity of Cells?

By lab analytical method: utilize qPCR – results similar to cell counts

- **Measures** – total number of cyanobacteria by measuring # of nucleic acid copies in a cell
  - Specifically measures # copies of 16S rRNA/mL, assumes 1 copy per cell
- **Results** – total cyanobacteria per volume (#/mL)
- **Examples of its use** - Faster & economical alternative to enumeration or cell density measurements by microscopy, forecasting, large batch processing
Lab Analysis – Quick Tips

- Maintain communication with lab, contact lab prior to sampling
- Identify what fraction of water sample should be tested - dissolved, particulate, or total fraction
- Request information about lab’s QA program
- Discuss how results are reported in lab reports
  - Reporting % error of toxin concentration is important when comparing results alongside action thresholds (e.g. results of 0.8µg/L ± 0.2 compared with 1µg/L threshold)

-Maintain communication with lab, contact lab prior to sampling to include recommendations into sampling design regarding: sample size, bottle type, sample handling and storage, turnaround times.
-For management decisions, consider the quality of data obtained by lab results.
SWAMP developed recommended quality control objectives and sample handling guidance for SWAMP monitoring projects. These guidelines are available to interested parties at SWAMP webpage

CA Cyanobacteria Lab Network - Consists of 5 regional labs and members from monitoring groups, agencies, and other interested organizations.
Additional Information

- Interested in contacting HAB researchers regarding genetic testing (i.e. toxin gene detection)?

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*Note - this is not an comprehensive list of researchers conducting research on genetic testing
References

- Fetscher, E. et al. 2015. Wadeable streams as widespread sources of benthic cyanotoxins in California, USA. Harmful Algae 49. 105-116.
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