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Cyanobacterial Harmful Algal Blooms: Chapter 20: Analytical Methods Workgroup Report

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Chapter 20: Analytical Methods Workgroup Report

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The topic of exposure assessment overlaps with other topic areas of this workshop. It includes considerations of establishment of long term monitoring and event response, sampling protocols, development and standardization of organism and toxin assays, funding mechanisms, and public outreach. The development of a coordinated infrastructure (funding, human resources, and facilities, materials and equipment) is key to successfully addressing the threat posed by CHABs.

The establishment of validated standardized protocols to detect cyanobacteria and cyanotoxins is of considerable importance given the increased occurrence of CHABs worldwide. Standardized methods are needed for studies assessing occurrence, monitoring and toxicity studies which are essential aspects of risk assessment and management and the development of guidance and regulation.

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Development of cyanobacteria and cyanotoxin standards for research and monitoring

There is a lack of reliable, quantitative standards for analytical determination of any of the toxins produced by cyanobacteria. Currently, while some of these toxins can be purchased commercially, availability and quantities are unreliable; and the identity and purity of the compounds is not guaranteed. Cases of either false identity or low purity standards have been documented in the scientific literature.

The criteria for selecting which toxins should be produced are:

1. Prevalence in US waters then global prevalence and
2. Documented risk of health effects (primarily irreversible human health effects, but also direct and indirect environmental impacts).

These toxins were discussed extensively during the ISOC HAB meeting. The toxins that need to be produced are microcystins, cylindrospermopsins, anatoxins (anatoxin-a, homoanatoxin-a, anatoxin-a[s]), saxitoxins (many of these are already commercially available at acceptable quality through shellfish monitoring programs), nodularin, and lipopolysaccharides. In addition to these, there are many unknown toxic and bioactive compounds that may become important in the future (Erhard et al. 1997; Cox et al. 2003; Berry et al. 2004). One example of this is BMAA (β -methylamino-L-alanine) which has recently been discovered to be present in many species of cyanobacteria (Cox et al. 2005).

Since microcystins have over 80 variants and congeners that vary in toxicity, it is recommended that several of the most prevalent variants are produced initially. These would be microcystin-LR, -YR, -RR, and -LA and their 3/7-demethylated analogues. Other variants of microcystin would be added to the list as needs arise. Since all of these toxins are derived from cyanobacterial cultures, care should be taken to insure sufficient quantities for monitoring and research purposes. One of the problems to overcome is ensuring cyanobacterial strain purity in order to maintain a consistent level of toxin production. An example of a well-characterized producer of seven microcystins is *Microcystis aeruginosa* M.TN-2 strain maintained in modified Fitzgerald media (Lee and Chou 2000). Algal cultures for production of large volumes of anatoxin-a[s], cylindrospermopsins, homoanatoxin-a must be identified and made available through culture collections. Optimal culturing practices need to be determined, particularly maximal toxin production as a function of temperature, light, and nutrient supply (Downing et al. 2005). Currently, there

are no practical and efficient synthetic methods for any of these chemical compounds except for anatoxin-a (Danheiser et al.1985).

Unlabeled and labeled stable isotope standards (for mass spectrometric work) are also needed. The first priority would be the production of standards of known concentration in solution, then neat, pure toxin and spiked matrices (i.e. cyanobacteria, shellfish, finished water, and food supplements). Extraction methodologies for optimal recovery need to be determined, and calibrated between laboratories, particularly for animal tissues during assessment of whole body burden. Standards should be certified for identity, purity and transport/long-term storage stability. Standards should be certified by gravimetric, NMR, and/or chemiluminescence nitrogen detection. For standards usable for biological research, biological activity information in a defined experimental setup should be included.

While there are no certified reference materials (CRMs) for any of these toxins (with the exception of the saxitoxin group), a number of companies are already pursuing this direction. CRMs of these calibration standards would be the ultimate goal. CRMs are used to evaluate the measurement precision and calibration of the laboratories and the analytical instruments used for toxin analysis. The consistency between laboratories and methods can be compared and authenticated by using CRMs.

Standards should be readily available and reasonably priced (non-profit preferred). When developing the structure for distribution of these standards they would be available in small amounts that could not be used for malicious purposes. Therefore, procedure for obtaining these standards should be kept straightforward to reduce paperwork and infrastructure.

The recommendations of the workgroup are to support these activities by pooling government agency, academic and international resources for the development of a reliable source of standards. Without such interactive support, gains in general knowledge, and managing or controlling CHAB will be slowed.

Sampling

To determine the occurrence and assess the risk of cyanobacterial harmful algal blooms (CHAB), it is important to collect samples that reflect the actual site or source conditions. Samples may consist of water, plankton, invertebrates, vertebrates, or sediments. Analyses may include toxins, genomic identification, enzyme or antibody assays, whole organism or tissue specific toxicity assays, or histopathology.

Lakes, reservoirs, estuaries, and streams are all potential sources of blooms. It is important to realize that the spatial and temporal distribution of any CHAB bloom is heterogeneous. Since CHABs growth rates range from 0.25 to 1.0 doublings per day, the field sampling efforts must consider growth rates of the cyanobacteria. Additional, care must be taken during the sampling effort, specifically with regard to altering the natural vertical distribution of cyanobacteria if surface scums are present.

The development of standard sampling procedures must be developed and validated. Aspects to consider include:

1. Safety protocols
2. Sample equipment (including cross contamination issues)
3. Field filtration
4. Sample stabilization (pH, temperature, light, control of degradation, etc.)
5. Sample transportation and storage
6. Sample documentation

Safety concerns during toxin collection must consider both short-term and long-term exposure hazards. A validated standard method for the field and the laboratory is necessary for collecting samples in a CHAB. Standard paraphernalia that should always be worn includes lab coats, gloves, masks, and goggles. Protocols for safe handling of fresh tissue, freeze-dried materials, including cell biomass, sediments, and neat toxins must be developed. General procedures used in Class II (biohazard) laboratories are recommended to minimize exposure risk to personnel.

Sample collection and preservation is dependent on the end use (i.e., toxin analysis, molecular experimentation, culture based, and tissues for histology). For example, culture based approaches require that the samples not be affected by perturbations, whereas, samples for toxin analysis requires different handling procedures. It is imperative that sampling techniques and equipment are used to minimize sample contamination (equipment, human, and cross contamination). For example, for many organic contaminants, glass and Teflon are preferable to plastics due to sorption and can impede cellular growth (if collecting for growth, this is important). Additionally, if the goal is to provide geospatial information (i.e., toxin abundance maps) the use of integrated versus discrete samples and fixed or randomly assigned locations may be used. There is, however, a need for standardized approaches for the analysis of whole water, particulate and dissolved toxins. Filtrate can be derived from a number of different filter

sizes (i.e. glass fiber filters of 1.2 or 0.7 microns, versus membranes of 0.45 or 0.2 microns). Tissue toxin analysis requires rapid preservation of samples, the ability to efficiently extract and quantify the toxin in light of differential matrix effects.

The collection of supporting data is critical to relate toxin abundance to physical and chemical causative variables. Physico-chemical parameters linked to cyanobacteria would include: dissolved-organic materials (DOM), pH, macro and micro-nutrients, temperature, turbulence, supporting plankton information including bacteria, algae, and zooplankton present, as well as light quality and quantity.

As with many other sample types, obtaining representative samples representative of the material/area being sampled, is paramount. The use of remote sensing to determine regions of interest may be useful in defining sampling efforts.

Sample processing and detection methods

Toxins produced by CHAB are of concern because of their demonstrated adverse affects to human health and the ecosystem. These compounds include the high priority toxins such as the microcystins (and nodularins), the cylindrospermopsins, and anatoxin-a, and many others including newly emerging toxins. Toxin-producing CHABs have been documented throughout the world and many regions of the United States. Simultaneous cyanotoxin profiling is a challenging area and even though we are not in the position to set limits at this point, current detection methods for each or some group of cyanotoxin, including screening and quantitative methods, hold promise for cyanobacterial toxin detection at the current WHO guidance levels (McElhiney and Lawton, 2005). There is, however, no suitable method to simultaneously extract and detect all the high priority cyanotoxins of interest to the Agency due to their biochemical differences. Routine monitoring is an unmet need in the US. Monitoring needs to be instituted and should address multiple facets including frequency of occurrence, performance of water utilities, transport within ecosystems, phytoplankton profiling, and toxin profiling.

Knowledge gaps in spite of the publication or availability of these methods, for regulation of the toxins, additional work is needed to support validation of sufficiently reliable and rugged methodology. This additional work will include development and field trials to address several important requirements. These include evaluating analyte stability, approaches and requirements for preconcentration (versus various sample matrix interfer-

ences) lyses methodologies of cells, and others. Analyte stability issues can be exemplified by the case of anatoxin-a which degrades rapidly at >7.5 pH, hence analytical samples must be maintained in acidic conditions. LC/MS methods detect the degradation products of anatoxin-a and anatoxin-a is easily mis-identified as phenylalanine. As part of sample preparation/sample extraction, differences in intra- and extra-cellular toxin concentrations must be considered. Intracellular concentrations, for example, relate potential toxicity in the case of cell lyses (due to processing or progression of the bloom).

Standard extraction procedures must also be developed. The following should be taken into account:

1. Solvent suitability for target toxins
2. Solvent suitability for multiple toxins determinations, where needed
3. Solvent disposal/safety issues
4. Protein binding (covalent and noncovalent) should be addressed
5. Other sample matrices such as biological tissues and sediment should be considered

Sample preconcentration must be addressed.

1. Choice of sorbent – to favor analyte versus matrix
2. Standardized protocols

Detection methods include:

1. Screening methods (assays such as ELISA, PP2A and other suitable methods)
2. Physicochemical (primarily separation) Methods (i.e., LC-UV, LC-Fluorescence, LC-MS/MS)

Total procedure time and complexity impact ruggedness, cost, and training needs and include:

1. Time to prepare sample (including extraction and cleanup)
2. Number and complexity of procedural steps (impacts difficulty and ruggedness)
3. Automation (column switching, robotics)
4. Data workup (software)
5. Total analysis time – considering above

Simple and reliable field testing methods are also needed. The most commonly used field methods are immunoassay-based tests (i.e., tube, microplate, strip formats). Commercially-available microcystin immunoassay kits are widely used to screen water samples for microcystins, with or without pre-concentration. Field methods are useful for quick screening of samples on-site, so immediate remedial actions can be taken and reduces the number of samples that require further analytical confirmation in the laboratory.

Cost is another important consideration and includes: initial instrument investment, kits and consumables, and labor costs. Safety considerations and restrictions (disposal, radiolabels, etc.) are also important.

A practical guide manual, “Toxic: Cyanobacteria monitoring and cyanotoxin analysis,” was just published commissioned and published by the European Community in September 2005 (Meriluoto and Codd 2005). This manual provides a comprehensive method for cyanobacteria and widely-studied cyanotoxins of interest. Methodologies for sampling and analytical methods are defined for many toxins in this publication and it would be useful to update this type of publication every 3–5 years with additional toxin methodologies and state of the art methodologies.

Setting Priorities

A critical component to the understanding of the potential for toxic episodes is an established program for monitoring and event validation. In its most basic format, monitoring must include tier-based approaches which are coupled to rapid and precise methodologies that can quantify toxin occurrence and/or measure biological effect. General field monitoring, incorporating remote sensing platforms (e.g., satellite imagery, deployed sentinel systems) need to be rapidly corroborated by laboratory analyses (i.e., toxin quantification, identification and culture analyses of cloned axenic organisms). The following outlines some identified abilities and areas of future prioritization for exposure assessment of cyanobacterial harmful algal blooms (CHABs).

Sentinel technology for CHABs includes “low” to high technology approaches.

1. Ground based sampling by volunteer groups has been an effective monitoring method in various rivers, lakes, and estuaries (VT, NY, and FL). This can be the first step in obtaining samples for characterization of any CHAB.

2. Remote sensing technology provides a method to identify blooms and appropriate sampling locations to assess CHAB. Remote sensing can use surface platforms: fixed or mobile units (such as Finnish use of ferries to sample the Baltic Sea, also Fig. 1 (see Color Plate 7)) or satellite imagery (e.g., Fig. 2 see Color Plate 7). Decisions regarding fixed sampling locations and use of drogue/physical circulation-driven sampling are essential to answer specific questions.
3. Remote sensing can take advantage of the presence of unique phycobiliprotein and carotenoid photopigments which provide distinctive markers for identification of cyanobacteria. The presence of coccoid cyanobacteria can be specifically identified by the presence of myxoxanthin, whereas aphanizanthin is diagnostic for filamentous cyanobacteria. Cyanobacteria can also be identified using specific absorbance characteristics of the phycobilins—particularly absorbance at 630 nm attributed to the phycocyanins (Fig. 1). The use of reciprocal reflectance data inversion can be used to accentuate spectral properties of interest. Equipment available for cyanobacteria detection includes a variety of hyperspectral sensors (see Ritchie and Zimba 2005, for a review of available sensor equipment and techniques for identification of various pigment signatures). Miniaturized dual spectral radiometers can provide a means of assessing total biomass and specifically cyanobacteria populations. This and other models offer the ability to simultaneously assess available light and algal reflectance, thereby allowing the use of all but highly transitional varying light conditions. One focus is the need to develop specific cyanobacterial reflectance models that are not ratio methods for estimating cyanobacterial biomass. Although these methods can be valuable, one cannot solely rely on satellite imaging data. Sometimes, cyanotoxins are present when there are no visible blooms.

Future Directions

One general goal is the development of a new generation of biosensors. Ideally these biosensors would be low cost, sensitive, reliable and relatively simple to use. Development of more extensive miniaturized biosensors will allow better cyanobacteria or cyanotoxin assessment. For instance, use of submersed hyperspectral radiometers coupled with biochip nanotechnology designed to assess cell surface recognition compounds, antibody coatings, and/or toxin recognition polymers will provide enhanced identification methods. These systems can provide sentinel type monitoring through fixed platforms, floating arrays, or on ships.

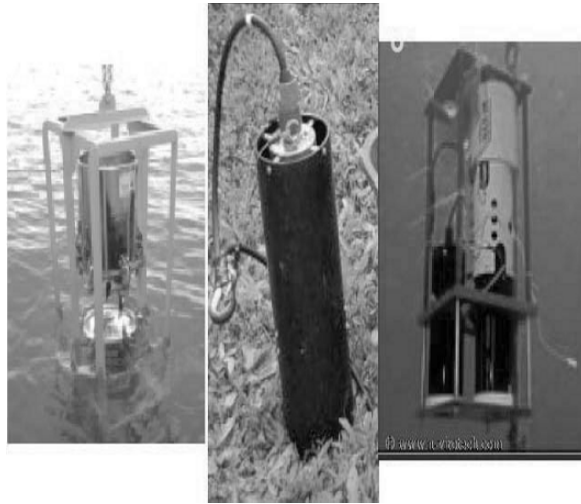


Fig. 1. Samples of currently available deployable systems that can be used in situ to sense conditions associated with potential CHAB events. 1). Flow Cam system from Fluid Imaging Technologies can identify cell type in situ. 2). Fluoroprobe system can identify water column phytoplankton based on a combination of 6 different fluorescence signatures. 3). NAS nutrient analyzer can detect in situ biogeochemical shifts that can be linked to pending CHAB events. (See Color Plate 7).

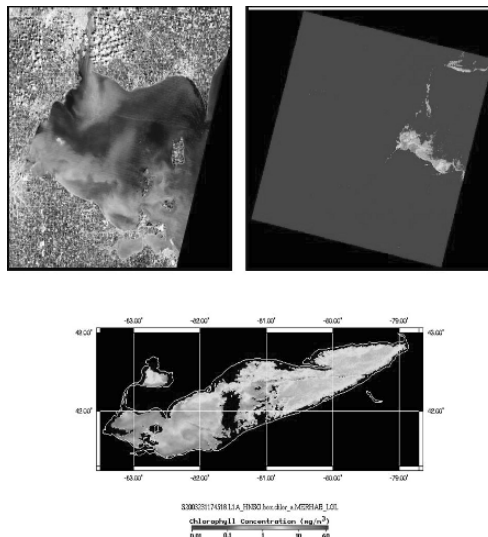


Fig. 2. Sample imagery available from satellites appropriate for monitoring CHAB events. True color imagery from Land Sat 7 (upper left, Rinta-Kanto et al. 2005) can be used to demonstrate potential algal blooms, which appear as green discolorations in the water column. The cyanobacterial-specific pigment phycocyanin can be elucidated from the appropriate applications of other algorithms (upper right, Vincent et al. 2003). Other imagery, such as daily Sea Wifs chlorophyll estimates (bottom) is available more frequently but provides less spatial resolution. (See Color Plate 7).

Color Plate 7

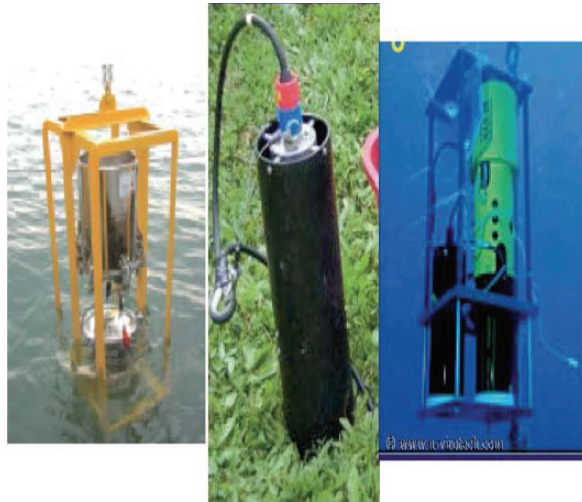


Fig. 1 (Chapter 20). Samples of currently available deployable systems that can be used in situ to sense conditions associated with potential CHAB events. 1). Flow Cam system from Fluid Imaging Technologies can identify cell type in situ. 2). Fluoroprobe system can identify water column phytoplankton based on a combination of 6 different fluorescence signatures. 3). NAS nutrient analyzer can detect in situ biogeochemical shifts that can be linked to pending CHAB events. (See page 477).

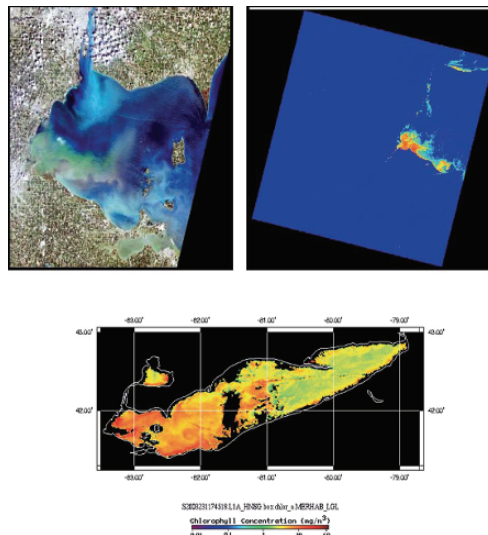


Fig. 2 (Chapter 20). Sample imagery available from satellites appropriate for monitoring CHAB events. True color imagery from Land Sat 7 (upper left, Rinta-Kanto et al. 2005) can be used to demonstrate potential algal blooms, which appear as green discolorations in the water column. The cyanobacterial-specific pigment phycocyanin can be elucidated from the appropriate applications of other algorithms (upper right, Vincent et al. 2003). Other imagery, such as daily Sea Wifs chlorophyll estimates (bottom) is available more frequently but provides less spatial resolution. (See page 477).

A critical need is increased knowledge of genetic markers for toxic cyanobacteria and their phenotypic expression:

1. Cyanobacteria identification is essential as a first step to provide firm bases for comparison of algal groups. This would include expanding our knowledge of the diversity of potentially important CHAB organisms and their associated biosynthetic pathways. Current efforts combining phenotypic appearance with molecular approaches will help unify taxonomic identification procedures (Komarek and Anagnostidis, 2005).
2. Support of culture collections with access by qualified investigators is one important mechanism for this task, as is support for genetic characterization and “classical” characterization of isolates.
3. The ability to identify toxin forming strains (e.g. *Lyngbya*, *Trichodesmium spp.*).
4. The ability to identify and characterize new toxins (e.g. BMAs, newly discovered *Trichodesmium* neurotoxin, other bioactive compounds).
5. Toxin biosynthetic pathways need to be elucidated. For example, although microcystin biosynthesis genes have been characterized, information on biosynthetic pathways for other CHAB toxins (i.e., cylindrospermopsin, anatoxin a, saxitoxin, BMAA) is largely unknown.

Proper sentinel deployment is also an essential need. Cyanobacterial blooms may occur on regional scales that are not easily detected by satellite imagery. Moreover, interference from atmospheric events (i.e., cloud cover, solar flares) can impede the ability of satellites to “see” events. Buoys/sentinel devices deployed *in situ* or on mobile platforms can avoid the atmospheric interference problems. Currently technologies are limited to fluorescence-based sensors targeting pigments similar to satellite systems. Future applications must move beyond this, and target the development of applications that can determine both cell type and cellular toxicity. Examples of similar systems include the Environmental Sample Processor (ESP) being developed at the Monterey-based Aquarium Research Institute (MBARI). Incorporation of emerging technologies into sentinel devices will allow for focused responses to events as they occur in real time.

Major future developments in the area of sentinel deployments will involve miniaturization of chemical sensory technology (i.e., hand held/deployable mass spectrometers) and will allow for real-time, on-line detection of toxins. Continued insight into the genetic mechanisms of toxin production, combined with advanced autonomous tools to characterize communities based on molecular markers, will allow for the determination of both the presence (DNA) and activity (RNA) of genetic systems capable of producing toxin production. Linkage of these systems to remotely deployable biosensors that can be incorporated into real time microsensors should allow for accurate characterization of cell abundance, toxin concentrations and toxin activity (Layton et al. 1998; Simpson et al. 2001; Yan et al. 2001; Mioni et al. 2003).

Specific priorities

1. Encourage interaction of CHAB researchers with marine scientists in order to effectively transfer existing remote platform and network technology to ongoing and future CHAB studies
2. Encourage widespread placement of remote sensors on available mobile platforms such as ferries, commercial and government over flights.
3. Encourage outreach programs to educate and recruit non-scientists as stakeholders
4. Support the development of sensor technologies; technologies with great promise include: microarrays; bioreporters, cytotoxicity monitoring, PCR technologies.
5. Encourage incorporation of microfluidics and nanotechnology into sensor development

Overarching considerations

CHAB events and impacts occur within the larger context of ecosystem processes. Therefore, the development of research strategies and activities should include consideration of complimentary and ongoing studies whenever possible. For example, since nutrient inputs influence cyanobacterial activity, site selection should favor areas with adjacent watershed studies or ongoing synoptic sampling and long term monitoring when possible.

CHAB issues fall within the mandate of multiple federal agencies (EPA, DHHS, DI, DC, DOD, DHS) as well as health and environmental agencies from the local to state levels). Therefore, an effective approach should be a coordinated program with funding and administrative support across interested agencies. A valuable component of many existing harmful algal bloom research programs is an outreach component, and this will also be necessary for a well-coordinated cyanobacterial research program. Outreach activities contribute to public awareness and support of research funding. They may also build the capacity of a widely distributed surveillance network to rapidly detect and response to the onset of CHAB events.

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