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Cyanobacterial Harmful Algal Blooms: Chapter 15: Cyanotoxins Workgroup Report

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Chapter 15: Cyanotoxins Workgroup Report

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Introduction

The Cyanotoxins Workgroup was charged with the identification and prioritization of research needs associated with: the identification of cyanotoxins; toxicokinetics and toxicodynamics of cyanotoxins; human susceptibility to the toxins; cyanobacterial genetics/omics and factors for inclusion in predictive models of toxin production; and risk reduction from an intentional or accidental release of cyanotoxins. Papers presented for the Cyanotoxins Session of the symposium on toxin types, toxicokinetics, and toxicodyamics (See Humpage this volume), cyanobacterial genetics of toxin production (See Neilan this volume), and parameters related to human risks from cyanobacterial exposure (See Love this volume) set the stage for Cyanotoxins Workgroup discussions.

A consensus was achieved regarding the need to focus on the major identified classes of cyanotoxins. The group expressed the belief that the most significant toxic components of presently occurring harmful algal blooms have been identified, and the knowledge gaps for these most prevalent toxins are great enough to warrant the attention of most of our

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future research. This belief does not negate the need to study mixtures of cyanotoxins and toxin precursors, especially those most likely to occur within a given bloom. Moreover, there is also a significant likelihood that novel cyanobacterial blooms and toxins will continue to emerge, and future identification of unknown bloom-forming species and their toxins will require ongoing diligence.

Charge 1: Identification

> Identify and prioritize research needs concerning the identification and quantification of cyanobacteria, cyanotoxins, and their toxicities.

The public health, environmental health, and economic impacts resulting from harmful algal blooms (HABs) create the need to prioritize research for the detection and identification of cyanotoxins. To comply with the Safe Drinking Water Act (SDWA), the U.S. EPA must identify contaminants that may require regulation in the future and periodically publish the resulting Contaminant Candidate List (CCL). The EPA identifies the data gaps associated with the contaminants on the CCL to prioritize research and data compilation to determine whether regulation of candidate contaminants is warranted.

In 1998 and again in 2005, EPA listed "Cyanobacteria (blue-green algae), other freshwater algae, and their toxins" on the CCL. The Workgroup felt that the CCL should be more specific; for example, listing the individual cyanobacteria and toxins of concern. Many genera and species of cyanobacteria are non-toxic; however, many other genera and species can produce a single known toxin and, conversely, a single species can produce a range of cyanotoxins. More detailed listings may evolve because the CCL is an iterative process that assimilates new data on contaminants as well as recommendations from authorized workgroups. More information can be obtained on the EPA CCL website, http://www.epa.gov/safewater/ccl/index.html.

Research Priority: Protocols for efficient production, certification, and distribution of pure toxins and standards of consistent, high quality.

Rapid and sensitive detection of cyanotoxins is an important goal for mitigating potential risks posed by HABs. Sensitivity and specificity of analytical methods for identifying the different classes of cyanotoxins and for accurate quantification depend on the availability of high quality toxin standards. The workgroup expressed concern that some published research was not as reliable as it might be because of reliance on in-house purification of toxins or use of commercial material that was later shown to be of poor quality. Standardized protocols are needed for the efficient production, certification, and distribution of pure toxins and standards. Commercial standards for cylindrospermopsin, some microcystins, nodularin, anatoxin-a, and saxitoxins are available (Table 1). However, concern regarding the reliability of some of the non-certified commercial standards has been expressed. Larger (mg) quantities of some cyanotoxins are available from certain research labs involved in cyanobacterial research, for example, the Australian Water Quality Centre (http://www.awqc.com.au/). Another concern is that recent regulations of high potency agents and toxins hamper the acquisition and storage of large toxin quantities that are needed for analytical methods development, validation, and, toxicological studies. Although safeguards are needed to prevent illicit uses of cyanotoxins, provisions for safe transportation and use in secure facilities are needed.

Table 1. Currently available cyanotoxin standards and certified reference materials (CRM)

Toxin type	Congener	Supplier	Catalog No.
Paralytic Shell-fish Poisoning	Saxitoxin dihydrochloride	Food and Drug Administration	Reference Standard Saxitoxin
	saxitoxin diacetate	National Research Council Canada (http://imb-ibm.nrc-cnrc.gc.ca/crmp/shellfish/psp_e.php)	CRM-STXdiAc
	saxitoxin dihydrochloride		CRM-STX-d
	neosaxitoxin	"	CRM-NEO-b
	decarbamoylsaxitoxin	"	CRM-dcSTX
	gonyautoxin 1&4	"	CRM-GTX1&4-b
	gonyautoxin 2&3	"	CRM-GTX2&3-b
	gonyautoxin 5 – aka B1	"	CRM-GTX5-b
	decarbamoylgonyautoxin 2&3	"	CRM-dcGTX2&3
	decarbamoylneosaxitoxin	"	CRM-dcNEO
	N-sulfocarbamoylgonyautoxins C1&2	"	CRM-C1&2
	Saxitoxin in acetic acid	Sigma-Aldrich	BCR663 CRM
	dc-saxitoxin	"	BCR543, BCR542 CRM
	neosaxitoxin	"	SO170
	saxitoxin diacetate salt	"	S1417

Toxin type	Congener	Supplier	Catalog No.
	Radiolabeled saxitoxin (^{3}H-STX)	Currently available at no cost through collaboration between FDA, NOAA and IAEA	
Microcystins	Microcystin LR	Alexis; Calbiochem; NRC Canada	350-012; 475815
	Microcystin LF	Alexis; Calbiochem	350-081; 475814
	Microcystin LW	Alexis; Calbiochem	350-080; 475818
	Microcystin RR	Alexis; Calbiochem; NRC Canada	350-043; 475816
	Microcystin-LR	Sigma-Aldrich	M-2912
	Microcystin LR	Cyano-Biotech	
	Microcystin LF	http://www.cyano-biotech.com	
	Microcystin LW	"	
	Microcystin RR	"	
	Microcystin YR	Alexis; Cyano-Biotech	ALX-350-044
	MC-7-desMethyl LR	NRC Canada	(see website)
	Nodularin	Alexis; NRC Canada	ALX-350-061
Other toxins	(+/-) –Anatoxin-a fumarate	A.G. Scientific	A-1065
	Cylindrospermopsin	National Research Council Canada, in collaboration with the Australian Water Quality Centre	CYN-CRM

This issue has also been addressed by other international organizations. A technical report available from the Organisation for the Prohibition of Chemical Weapons (OPCW) website (http://www.opcw.org/html/global-/s_series/98/s78_98.html) indicates that international access to tritiated saxitoxin is extremely limited, and that most nations do not have access to expensive HPLC systems for monitoring saxitoxin levels. Increased availability and distribution of standards, in addition to inexpensive monitoring and detection systems, are urgently needed to monitor cyanobacterial toxins. The UN Food and Agriculture Organization (FAO) also addressed this issue. "It is possible to measure PSP [paralytic shellfish poisoning] compounds by a number of analytical-chemical methods but they all have some limitations, and they often cannot easily be operated because of the lack of reference materials, although recently some progress has been made in this area. However, they are expensive and mainly available from one source. The efforts undertaken by the European Commission's SMT Programme have led to shellfish reference materials with certified mass fractions of some of the toxicologically most significant PSP toxins. Despite these positive developments, the analytical situation remains difficult and the lack of pure PSP compounds in sufficient quantities for repeated dose toxicity studies is a limiting factor in the development of reliable risk assessment." (Marine Biotoxins, FAO Food and Nutrition Paper Number 80, 2004). The recent Harmful Algal Research and Response plan (HARRNESS 2005) also listed establishment of reference material infrastructure and improved availability and distribution of toxins and their metabolites as top priorities.

The workgroup concluded that reliable, well-characterized standards are needed for as many of the common toxins and congeners as possible so that research results from different laboratories can be reconciled. The lack of pure toxins and standards not only limits monitoring efforts, but the ability of investigators to conduct the chronic, low-dose toxicity studies required for risk assessment. Currently it is inadvisable to have detailed protocols for the production of cyanotoxins widely distributed, as these could be used as recipes by bioterrorists. One option for safely acquiring adequate quantities of standards for research is to designate a few reputable and qualified laboratories as infrastructure resources for producing, certifying, and distributing specific chemical groups on an "at-cost" basis. These services must be provided at a cost that is not prohibitive of their use. In-house production by individual investigators was not the preferred option due to the resource and economic drain that would impede research efforts, and because of the lack of ability to fully characterize the material produced.

Standards for four groups of toxins were designated high priority: microcystins, cylindrospermopsins, anatoxin-a, and saxitoxins (i.e., PSPs). All four toxin groups are relevant to the U.S. EPA because they are highly distributed in the U.S. All four groups can impact drinking water reservoirs, farm irrigation, and recreational activities in freshwater environments.

Dangerous Goods Regulations and the Transport of Cyanotoxins

Cyanotoxins come under Dangerous Goods (DG) regulations for transport of hazardous materials (Metcalf et al. 2006). These regulations are defined under UN and IATA frameworks, but many countries have their own specific regulations as well, particularly for control of import and export. By law, such materials must be packed for transport by people specially trained and licensed for DG handling to ensure compliance with DG transport regulations. Freight companies will not accept these materials unless a licensed DG handler certifies that the package and labeling comply with the regulations. Material Safety Data Sheets specific to the DG must accompany the package. Only a few freight agents routinely transport DGs around the world. Most of them employ Custom's Brokers to facilitate importation into the destination country. It is important to use one of these companies to avoid delays, despite the added cost. It is also important to obtain legal opinion to ensure compliance with the plethora of DG and other relevant regulations, particularly for saxitoxins and microcystins because of the added Chemical Weapons Convention (CWC) regulations that apply to these toxins. Australia's CWC regulations require an export permit and quarterly reports on toxin use from the recipient. Although significant effort is required to develop this capability, it is no longer acceptable to simply mail "research materials" without taking DG and other regulations into account. These requirements will limit the number of labs that find it worthwhile to produce and supply cyanotoxins, again pointing to the need for infrastructure resources for production, certification, and distribution.

Developing Standard Methods to Separate and Identify Toxic Components of Raw Water and Crude Extracts

Prior to identifying research needs concerning the development of analytical methods to identify cyanotoxins, the workgroup recognized several overarching considerations and discussed current methods development projects. Methods are needed to identify the cyanobacterial species that comprise a bloom, as well as to identify individual cyanotoxins within the

bloom. Additionally, it is important to develop methods that discriminate between cyanobacterial strains and species that are capable and incapable of producing cyanotoxins that may pose risks in both recreational and drinking waters.

Microscopy or morphology-based monitoring is the method traditionally used to identify potentially toxigenic cyanobacteria. A monitoring or screening process has been instituted in Australia to monitor sources of drinking water, whereby the presence and quantity of cyanobacterial cells are identified based on microscopic observations of water samples. When cyanobacteria are present in significant numbers ("Alert Levels"), additional biochemical and molecular assays further characterize the cyanobacteria present. If toxicity screening assays are positive, additional treatment processes are included to mitigate the likelihood of toxins persisting through to the finished water. This approach relies on cellular morphology to identify species that can potentially produce toxins; however, this method does not determine whether a particular toxin is, in fact, present.

Standard methods exist for the separation and identification of many cyanobacterial toxins (for examples, see Meriluoto and Codd 2005, Moore 1996, WHO 1999 [http://www.who.int/docstore/water_sanitation_health-/toxicyanobact/]). However, there is continuing need to develop assays for newly discovered toxins, for methods refinement, and for validation and certification by organizations such as the AOAC International, a non-profit association of analytical communities. A current project is refining existing methods and developing new methods to support the collection of cyanotoxin occurrence data in cells and surface water. The project, entitled "Determination and Significance of Emerging Algal Toxins (Cyanotoxins)," is sponsored by the American Water Works Research Foundation (AwwaRF Project 2789, http://www.awwarf.org/research/TopicsAnd-Projects/projectSnapshot.aspx?pn=2789).

As better analytical methods have been developed in the shellfish industry (for example more sophisticated mass spectrometry), more toxin analogs have been detected. However, since toxicological data are unavailable for these compounds, regulators cannot use an evidence-based approach for toxicity classification. It is therefore important that toxicological characterization keeps pace with detection of new compounds. An alternative approach is further development of a structure-activity classification system. Less satisfactory is use of the default assumption that toxicity of an analog is equivalent to that of the most toxic known congener.

Emerging and novel cyanobacteria and their toxins. The workgroup considered the need for field ready, reliable, and inexpensive methods to detect currently known, common cyanobacteria and their toxins to be a

higher priority research need than the identification of novel cyanobacteria and cyanotoxins. The workgroup agreed that the major cyanotoxins which pose health and ecological risks have been identified. Emerging toxins were considered of somewhat lesser importance than the need for improved methods to assess known organisms and toxins. Historically, new toxins have been found due to accidental poisoning of wild, farm or domestic animals (or in the case of cylindrospermopsin, humans), rather than through formal surveys. Better links with veterinarians and National Parks staff, for example, would facilitate this process.

Complex mixtures. The identification and prioritization of research needs concerning cyanotoxin identification and quantification represents a unique challenge because cyanobacterial toxins generally occur as mixtures. The toxins usually occur as mixtures of analogs of the same toxin type (for example, there are about eighty known microcystins) and/or mixtures of different toxin types (for example, microcystins mixed with cylindrospermopsin. Toxicity screening assays are required to determine the total toxicity of mixtures present in blooms because all mixture components are not likely to be identified in toxin identification assays.

Synergisms in complex mixtures. Another aspect of the "known toxin" and "new toxin" issue is the observation that toxicity assessments of crude cell extracts usually indicate greater toxicity than can be attributed to the known toxins within the mixture. Similar patterns appear in many studies of chemical ecology (Paul et al. 2001, Pietsch et al. 2001). Although in some cases this situation can be taken as evidence of undiscovered toxins, the possibility has also been raised that there are compounds produced by cyanobacteria that, although not toxic themselves, can modify the effect of the known toxins. These could be non-toxic analogs of known toxins. This issue has received little research attention thus far. Furthermore, the list of non-toxic but biologically active compounds known to be produced by cyanobacteria is continually growing (see Table 1 in Humpage this volume).

Because compounds present in minor concentrations may have additive or synergistic effects with the pure toxins of interest, a clear research goal is to identify compounds in the crude extracts that enhance the toxicity of pure compounds, and to identify whether any compounds in crude extracts can reduce the toxicity of pure compounds. Therefore, assessments of one or several toxins may not be sufficient to characterize the risk posed by the mixture of cyanotoxins in a bloom. This issue is of particular importance to the design of toxicological experiments whose aim is the provision of data to support regulatory limits for the toxins in drinking water. These ex-

periments are universally done using pure toxins, but some consideration should be given to the inclusion of at least one treatment group receiving a crude extract for comparison. However, if such experiments demonstrate these hypothesized effects, then the range of compounds requiring detection may multiply considerably.

Research Priority: Expand the use of toxicity screening assays with a focus on pathology-based and mechanism-based screening

Pathology-based and mechanism-based toxicity screening

Due to the lack of readily available, validated, analytical methods that are capable of detecting the range of cyanotoxins known to exist, and of reference standards for them, the workgroup prioritized the expansion of toxicity screening assays with a focus on pathology-based and mechanism-based screening assays. Several approaches were suggested: 1) *in vitro,* utilizing cell-based assays and molecular techniques; 2) *in vivo,* such as the zebra fish egg test and various crustacean-based assays; 3) developmental screens involving fish or mouse embryos and; 4) assays that account for temporal effects. Screening assays should be as simple and sensitive as possible. Additional considerations are cost per assay and required technical expertise and resources. Screening assays may be used to detect toxins during occurrence surveys, to determine the environmental effects of the toxins, to determine the environmental factors that influence toxin production, and to monitor water bodies used for drinking water. Each of these uses will require the selection of an assay appropriate for the particular use and research need.

As previously mentioned, the basic question of how to deal with mixtures arose as a major complicating factor in developing cyanotoxin screening methods. Other factors to be considered are the effects of degradation rates, pH, photolysis, and total organic carbon (TOC) on the assays. Additionally, effects of toxins may have a temporal aspect that is not easily addressed by short-term *in vitro* assays. The workgroup agreed that the advantage of toxicity screening methods is that they measure toxicity and do not just identify individual toxins. This distinction is important because of the enormous toxicity differences between congeners. Cyanobacteria frequently lose the ability to produce certain congeners during laboratory cultivation. Therefore, it should be anticipated that results from field screening assays may differ from the more advanced laboratory analysis with cultured cyanobacteria.

Based on the Organisation for Economic Co-operation and Development (OECD) Guideline for Testing Chemicals-Direction 210, the zebra fish egg test was proposed as a rapid and sensitive screening tool for evaluating cyanotoxicity with relevance to understanding the environmental effects of these toxins. Ongoing studies are evaluating the effects of cyanotoxins (purified and raw extracts) on zebra fish, and a future assessment of these studies will help in evaluating the utility of this approach as a screening tool for cyanotoxicity. Assays based on transfected mammalian cell-lines that rapidly detect the characteristic biochemical effects of the cyanotoxins are also being developed. It is expected that these will provide a diagnostic capability that will help identify the toxin(s) involved.

Biochemical assays

Biochemical assays have been published for a number of cyanotoxins. Examples include protein phosphatase inhibition assays for microcystins and a protein synthesis inhibition assay for cylindrospermopsin (CYN). Sigma has developed a Protein Tyrosine Phosphatase (PTP) assay kit which can be used to screen for PP1 inhibitors, while Biosense (www.biosense.com) has a test kit in development that uses an immobilized PP2A active site as the receptor in a competitive binding assay micro-plate format.

Antibody-based assays exist for the PSPs (Jellett Rapid Test for PSPs and Ridascreen ELISA for saxitoxin), but little work has been done so far to prove their efficacy for use with the range of congeners produced by freshwater cyanobacteria. Established ELISA kits are available for microcystins. Various products have been evaluated previously (see AwwaRF report 2789). An ELISA for CYN has recently been announced by Abraxis. Biosensors embedded in semiconductor microchips may provide an alternative means of detecting the presence of cyanotoxins, instead of the current reliance on HPLC and ELISA-based assays. Biochips for saxitoxin have already been developed, for example Dill et al. (2001). This publication demonstrates the ability of CombiMarix Corporation's microarray antibody chip to detect saxitoxins (http://www.combimatrix.com/). Aptamers specific for microcystin have been developed but assays based on them do not yet have the required sensitivity and specificity. Assays based on artificially produced antibody fragments are also at the experimental stage. Although the sensitivity of biochips operating in reservoirs and water treatment facilities remains to be determined, the development of these biosensors represents an important area for future research. A drawback of all antibody-based technologies is that affinity for the toxins does not correlate with toxicity, and new analogues may not be detected even though they are toxic (see AwwaRF report 2789). The development

of a biosensor to assay only for a particular cyanotoxin would preclude the identification of the contaminating cyanobacteria since multiple genera may produce the same toxin.

Availability of antibodies

There are no readily available non-proprietary antibodies for saxitoxin (STX) or any of the other cyanotoxins, although a number of laboratories around the world have developed or are in the process of developing antibodies for use in their own research or assay development. Proactive steps should be taken to link these laboratories with kit makers so that assay development can be accelerated. The development of reliable assays is also contingent upon a previously identified priority – enhancing the supply of reliable chemical standards.

Research Priority: Develop rapid PCR-based assays for the presence of specific cyanobacteria and the potential for production of specific toxins

Molecular-based monitoring

The *in vitro* molecular approaches based on the polymerase chain reaction (PCR) have advantages as screening tools for the presence of toxigenic species because they have the potential to yield more rapid and more sensitive results (See Charge 5, below). To select appropriate genetic markers, the Workgroup agreed that more genetic information is needed. There are currently no whole genomes available for any toxin-producing cyanobacteria. Although the microcystin B biosynthetic gene cluster (*mycB*) is known, those encoding other cyanotoxins such as PSPs and cylindrospermopsin are not yet known. Molecular methods for assessing potential toxicity are limited by this lack of information.

Generally, the technical expertise and resources required for PCR-based techniques are greater than that of a trained microscopist who identifies cyanobacterial cells, but are less than that of an analytical chemistry lab employing HPLC identification methods and that of a lab conducting toxicity screening assays. Established techniques exist for using the genetic sequence of the 16S ribosomal RNA subunit to identify species of cyanobacteria. Some laboratories are developing assays that use the presence of toxin biosynthetic genes, such as *mycB*, to judge whether a bloom has the potential to produce toxins. Every strain of cyanobacteria with the gene for toxin production is capable of producing toxins, but the conditions for toxin production may not be present. Conversely, if the cyanobacteria lack

a gene for toxin production, no toxins will be produced. To identify both toxicity genes and individual cyanobacteria, molecular screening assays should include toxin producing and genera- and species-specific biomarkers. Historical occurrence data, once developed, is invaluable in selecting appropriate markers. However, ecological theory suggests that as a region's environmental conditions change, the organisms found in that region will also change. This process is hypothesized to account for the northward spread of *Cylindrospermopsis raciborskii* that has been observed as global warming continues.

A key development in our ability to predict the occurrence of C-HABs has been the identification of the microcystin gene cluster (*mycB*). This gene can be used as an indicator of potential toxin production, alerting water quality managers to the possibility of a toxic bloom. Reservoirs can be monitored by extracting DNA from whole algal communities, and testing for the presence of the microcystin gene by a PCR-based assay (e.g., Hisbergues et al. 2003). A test for CYN-producing genes has also been published (Fergusson and Saint, 2003). Similar tests would give additional warnings for the potential presence of other toxins, including saxitoxin and anatoxins. However, the biosynthetic genes responsible for the production of these latter toxins are currently unknown. In addition, genes that interact with the toxin gene clusters remain to be fully described in *Microcystis*, and are completely unknown for the other organisms. Whole genome sequencing, followed by bioinformatic analyses, is another approach that can be used to search for toxin gene clusters (see Charge 5 for a detailed discussion).

Research Priority: Develop molecular and toxicological fractionation methods to identify toxic components of raw water

As described earlier in this report, many compounds might act synergistically to augment the effects of cyanotoxins. Crude extracts from blooms often are more toxic than can be accounted for by their content of the purified component toxin(s). This enhanced toxicity may be due to interaction with other (known or unknown) cyanotoxins or because there are other compounds that enhance toxicity. These compounds may include water treatment byproducts, environmental/UV degradation byproducts, other toxic components in raw water, and bacterial metabolites. Because our current knowledge of cyanotoxin interactions with other contaminants is limited, there is clearly a need for continued toxicity testing of any potential byproducts produced by treatments to improve water quality. Simi-

larly, we know very little of the reactive chemistry of cyanotoxins in matrices, including their degradation chemistry and their physical and chemical reactions with sediment and other complex matrices.

The approach of comparing the toxicity of crude cell extracts with that of purified toxins has been undertaken by several labs. Such comparisons performed *in vitro* using cell cultures (preferably of human origin) are needed. One hypothesis is that these unidentified compounds are analogues of known cyanotoxins. This hypothesis should be evaluated in organic chemistry labs with an interest in secondary metabolites. The toxicity of novel analogues should be assessed in isolation and in combination with known analogues. Immunoaffinity is an approach for distinguishing the effect of cyanotoxins from that of other compounds. This approach was applied to microcystin. The toxin was removed by binding to an immunoaffinity column containing antibody to microcystin (Kondo et al.1996), followed by toxicity assays of the separated eluents. This approach could be expanded to cylindrospermopsin-producing cultures or blooms once suitable antibodies are available. An advantage of this method is that the bound toxins can be easily recovered.

Non-mammalian model systems that have provided information of relevance to public health risk assessment include *Xenopus* oocytes and zebrafish. Although these models aid our understanding of effects of cyanotoxins in the environment, any effects must ultimately be confirmed in at least one mammalian model system to assess risks to human health. Since *in vitro* experiments are limited by experimental constraints and difficulty of interpretation, it will be necessary to use mammalian model systems to confirm all hypothesized mechanisms of action.

The above discussion notwithstanding, research prioritization requires some knowledge of the primary risks in a particular country or region. Established detection methods should be implemented to provide local cyanobacteria and cyanotoxin occurrence data. This approach focuses on common analogs, but substantial differences in toxicity or chemistry between the identified and unidentified analogs could lead to inaccurate assessments of risk. A comparison to the toxicity caused by the identified analogues and crude cell extracts can be used to assess the overall risk. Thus, the assessment of risk in local and regional areas may benefit from an on-going prioritization process.

Characterization of unknown toxic compounds

There is a high likelihood that novel cyanobacterial blooms and toxins will continue to emerge. A key research priority will be the identification of unknown bloom-forming species and their toxins. If a new, emerging

cyanobacterium (e.g. *Lyngbya* cf. *confervoides* on the coast of Florida), produces unknown toxins, standard chemical and toxicological methods are needed to separate and identify the toxic components. Several labs working on natural products from cyanobacteria have established standard procedures for cyanobacterial culture, activity assay-based fractionation, and LC, MS, and NMR of unknown compounds. These processes have proven fruitful in the past (see Table A.1 in Humpage this volume, and references cited therein). These studies generally provide enough chemical information to enable the development of methods to identify the compounds. Although initial *in vitro* cellular or enzyme inhibition data may be obtained, further toxicological characterization of the compounds is needed to produce meaningful risk assessments.

Recommendation: Develop more local expertise to identify cyanobacteria and their toxins. Several symposium attendees stated that their local water quality management teams do not have the expertise to identify cyanobacteria or cyanotoxins. Education of local water quality agents will be needed to ensure that blooms of toxic organisms will be detected. In addition, simple and fast procedures for toxin detection need to be distributed to local agencies. Although ELISA-based detection kits are already available for microcystins, cylindrospermopsins, and saxitoxins (e.g. from R-Biopharm), some local agencies were not aware of their existence. Outreach and education to local water quality agencies will be needed to implement any findings and recommendations of a national research plan. The development of predictive models for bloom and toxin occurrence is a long-term goal.

Charge 1: Identify and prioritize research needs concerning the identification and quantification of cyanobacteria, cyanotoxins, and their toxicities.

Near-term Research Priorities

- Developing protocols to ensure the availability of reliable reference standards of cyanotoxins as well as antibodies for use in biochemical assays
- Developing local expertise to identify cyanobacteria and their toxins
- Developing PCR-based screening assays to identify cyanobacteria and the potential for toxin production; develop an ELISA assay for anatoxin-a

- Developing ways to increase the rapidity, sensitivity, specificity, and robustness of biological toxicity screening assays
- Obtaining ecological and genetic information on mechanisms of toxin production and toxin regulation.

Long-term Research Priorities

- Developing biologically-based in-line, real-time biosensors for automated water quality monitoring
- Assessing the interactions of cyanotoxins with complex mixtures and complex matrices, including identification of transformation byproducts of toxins resulting from water treatment and environmental or UV degradation
- Developing a predictive model of the occurrence of C-HABs and toxic blooms.

Charge 2: Toxicokinetics

Identify and prioritize toxicokinetic research needed to improve human health risk assessments.

Toxicokinetic research on cyanotoxins to date has been limited to basic studies of tissue distribution/elimination and initial efforts to characterize primary metabolism of the more prevalent toxins. Determinations of the relative contribution of different exposure routes to total exposure, the role of metabolism in toxic responses and detoxification, and particularly, the characteristics of human toxicokinetics, are important endeavors for future research.

Research Priority: Labeled compounds and antibodies are needed for Toxicokinetic studies

Microcystins (MC) have been radioactively labeled in individual laboratories as needed for research. The only exception is ^{3}H- microcystin (Robinson et al. 1989) that was labeled by Amersham for the Pathophysiology Division, United States Army Medical Research Institute of Infectious Diseases, Ft Detrick, Md. Brooks and Codd (1987) grew *Microcystis* in

culture with sodium ^{14}C-bicarbonate. This required exposure to radioactivity for long periods, and resulted in a labeled product with low specific activity. The method used by the majority of researchers for labeling MC is reduction of the methyl dehydroalanine residue by NaB^3H_4. The products of reaction were characterized by Meriluoto et al. (1990) as retaining the toxicity of the native MC-LR with somewhat less potency. This labeled compound differs from native MC by not being able to form a covalent bond with protein phosphatase 1 and 2A (PP1 and PP2A): the targets of MC in cells and *in vivo*. Falconer et al. (1986) and Runnegar et al. (1986) labeled the tyrosine of MC-YM enzymatically with iodine 125 and showed that the monoiodinated product of the reaction retained the same toxicity in mice as the native MC-YM while the di-iodinated product had somewhat less potency.

There is only one report of a labeled cylindrospermopsin. This derivative resulted from the growth of the cyanobacterium with sodium ^{14}C-bicarbonate: (Norris et al. (2001). Radiolabeled saxitoxin (^3H-STX) is currently available at no cost through a collaboration between FDA, NOAA and IAEA.

Many antibodies have been prepared commercially that are incorporated into ELISA kits for the detection of MC in water. Research groups have also developed antibodies to MC for use in their laboratories. Commercial MC monoclonal antibodies are marketed by Alexis. Dr. Michael Weller (Technische Universitat Munchen, Institut fur Wasserchemie) has investigated the specificity of these antibodies towards MC-LR and other congeners providing a good basis for their potential scientific use. Nevertheless, the specificity of most of these antibodies needs further characterization. The degree to which antibodies detect MC when it is covalently bound to the protein phosphatases or tightly bound in the inhibitory complex is also unknown. Matrix effects also have not been evaluated. Antibody detection of congeners, metabolites, and degradation products following water treatment also requires study. Metcalf et al. (2002) compared the cross-reactivity of MC-LR, conjugates of three commercial ELISA kits, and an-in house ELISA. Cross-reactivity for the compounds tested depended on the solvent used. Antibodies are used in the ALGAETOX project for the development of biosensor systems to detect and quantify algal blooms and toxins (TU Berlin, Fachgebiet Landsschatokologie, insbesondere Oko-toxikologie). With these caveats, antibodies have been used to detect MC in tissue/cells for immunochemical analysis and immunohistochemical localization as well as for some quantitation.

A new ELISA kit for the detection of cylindrospermopsin via antibodies is now available from Biosense Laboratories. The antibody binds to CYN with no cross-reactivity with other non-related toxins or compounds. The

assay has a detection range between 0.05 and 2.0 ppb and can be used with a variety of environmental samples such as water and fish tissues. Abraxis recently (Jan 2007) announced production of a CYN ELISA, but it is not known whether the antibodies are available separately.

The production of stable standardized radiolabeled toxins and antibodies with known specificities are important near-term needs for toxicokinetics research.

Research Priority: Classical toxicokinetics (distribution, half-life, elimination, etc.) in laboratory animals

Studies employing radiolabeled microcystins have demonstrated that the liver is the main site of toxin accumulation (after oral, intraperitoneal, or intravenous dosing) and that hepatic toxin concentrations remain relatively constant for up to 6 days after treatment. These findings include: 30-40% of ^{125}I-MC-YM dose in liver at death or 24 hr after i.p. injection (Runnegar et al 1986); 76% of ^{14}C-MC-LR dose in liver at 1 min and 77% in liver at 60 min after i.p. injection (Brooks and Codd 1987); 56% of ^{3}H-MC-LR dose in liver at death or 6 hr after i.p. injection (Robinson et al 1989); 30% of ^{3}H- dihydro MC-LR dose in liver at 45 min after i.v. injection (Meriluoto et al 1990); 67% in liver of mice injected i.v. after1 hr and 6 d (Robinson et al 1991); and 47-65% of ^{3}H- dihydro MC-LR dose in liver at 5 hr after i.v. injection of pigs (Stotts et al 1997). Pretreatment with rifamycin (inhibitor of MC uptake in hepatocytes) decreased the hepatic MC concentration after dosing and protected from MC toxicity if given 5 min before MC dosing (Runnegar et al. 1993).

Small amounts in the kidneys, intestine, and carcass indicated that these differently labeled MCs all concentrated similarly in the liver, and that there was no significant loss of label from MC. Mice had excreted 9 and 15% of an i.v. dose in the urine and feces after 6 days, respectively. Although none of the labeled microcystins are ideal (see Meriluoto et al. 1990 for a discussion), they all have similar organotropism, and subsequent work from many laboratories with animals, perfused livers, and isolated hepatocytes confirmed the findings.

Microcystin antibodies have been used for MC quantitation in tissues of treated mice and fish: (Liu et al. 2000; Guzman et al. 2003). Immunostaining and semi-quantitative analysis in mice were reported by: Yoshida et al. (1998), i.p. mice, liver accumulation; Yoshida et al (1997), oral in mice with liver accumulation; Ito et al. (2000), oral dosing with liver accumulation in mice receiving a lethal dose. Oral dosing showed some additional MC staining in the intestine. Guzman et al. (2003) reported MC staining

in the nucleus as well as cytoplasm of hepatocytes in a section of mouse liver. Liver accumulation was also shown in rainbow trout (Fischer et al. 2000).

Table 2. Tissue Distribution and Elimination of Radiolabeled Microcystin-LR in Mice

Species & Route	Dose & Isotope	Time	Tissues	% of Dose	Half Life
Mice i.p.	45 µg/kg Sublethal &100 µg/kg Lethal	6 hrs or death	Liver Kidney Intestine Carcass	>50 1 10 10	
Mice i.p.	70 µg/kg	60 min	Liver Kidney Intestine	60 1 9	29 min
Male Mice i.v.	^{3}H MYC-LR 35 µg/kg Sublethal	1 min	Liver Intestine Kidney Carcass Plasma	23 5 2 30 25	α-phase: 0.8 min β-phase: 6.9 min
Male Mice i.v.	^{3}H MYC-LR 35 µg/kg Sublethal	60 min	Liver Intestine Kidney Carcass Plasma	67 9 1 6 trace	
Male Mice i.v.	^{3}H MYC-LR 35 µg/kg Sublethal	6 days	Urine Feces Liver	9 15 67	Covalently bound in liver cytosol: Day 1 - 83% Day 6 - 42%

Adapted from Robinson et al. (1989, 1991)

^{14}C-labeled cylindrospermopsin was administered i.p. to mice resulting in excretion of CYN mainly in the urine but also in the feces (Norris et al. 2001). The excretion patterns showed substantial inter-individual variability between predominantly fecal or urinary excretion, but excretion patterns were not related in any simple manner to toxicity. The authors also found CYN in the liver: 21% of dose at 6 hrs and 13% at 48 hr. Additional evidence indicated CYN or a metabolite was tightly bound to protein. Analysis by HPLC indicated that this tightly bound CYN was in part a hydrophilic metabolite with a different elution time. This single report shows the complexity of CYN distribution, metabolism and excretion. Much

more work is needed to confirm and extend the findings particularly given the intra-mouse variability reported.

Toxicokinetic and toxicodymanic studies have been performed for saxitoxins (STXs) in cats (Andrinolo et al. 2002). Oral doses of gonyautoxins 2/3 were administered to cats to determine how toxins are absorbed in the digestive system, where they are absorbed, the absorption rate, the maximal concentration in the plasma, and the time needed to reach that maximum. Oral uptake was efficient, toxin distributed rapidly throughout the body (including the brain), was cleared by simple glomerular filtration, and there was no evidence of metabolism of the toxins. Urine appears to be a major route of toxin excretion in humans (Gessner et al. 1997). The toxicokinetics of saxitoxinol in rats has also been investigated (Hines et al. 1993; Naseem 1996).

β-Methylamino alanine (BMAA) toxicokinetic studies in rats and monkeys demonstrated rapid and virtually complete uptake via the oral route, and distribution throughout the body (Duncan et al. 1991; 1992). The brain contained only about 0.08% of the administered dose at 48 hrs. There was evidence of active transport across the blood-brain barrier via the large neutral amino acid carrier (Km=2.9mM), but this would not be rapid when BMAA is in competition with normal levels of natural amino acids (Duncan et al. 1992; Jalaludin and Smith 1992). Approximately 1.4% of an oral dose, and 1.8% of an i.v. dose, were excreted unmetabolized in the urine by 48 hrs, whereas approximately 22% could be accounted for in total as either unmetabolized or acid hydrolysable (Duncan et al. 1992; Jalaludin and Smith 1992; Humpage this volume). L-amino acid oxidase has been shown to metabolize BMAA, eventually leading to N-methylglycine, but the rate appears to be quite slow (Hashmi and Anders 1991). The racemate and L-forms of BMAA caused cerebellar dysfunction and degeneration of GABAergic cells, presumably through excitotoxicity, but multiple low doses of racemate did not shown evidence of cumulative toxicity (Seawright et al. 1990).

Transport

Microcystins. Microcystins require transport via specific organic anion transport proteins to cross cell membranes (Runnegar et al.1993; Runnegar et al. 1995a; Fischer et al. 2005). Many studies have shown uptake of MC by active transport in hepatocytes but not in other cells including hepatic nonparenchymal cells. There is a good correlation between MC accumulation and toxicity. Hepatocytes from rats and mice as well as from many aquatic animals have been studied. The organ specificity of MC is due to transporters that predominate in the liver. MCs can inhibit protein phos-

phatase activity with similar potency in cell/tissue extracts from any eukaryote tested so far. The uptake of MC is inhibited by bile acids and dyes. Perfused livers and whole animals were protected from toxicity if pretreated with these compounds.

Very recently the transporters that mediate MC uptake were identified, opening the possibility for many new studies (Fischer et al. 2005). *Xenopus laevi* oocytes were injected with complementary RNA of the known multi-specific organic anion transporting polypeptides (OATPs), and uptake of ^{3}H-dihydro MC-LR was measured. Three human OATPs were shown to mediate the transport of MC: OATP1B1 (was OATPC), OATP1B3 (was OATP8) and OATP1A2 (was OATPA). The first two are mainly expressed in liver, thus explaining the MC uptake inhibition in hepatocytes by bile salts and other compounds (substrates or inhibitors of OATPs), as well as the protection by predosing with rifamycin *in vivo* (Runnegar et al. 1993). The third transporter is also expressed at the blood-brain barrier. This finding indicates the possibility of MC uptake by the brain. Brain uptake of MC by OATP1A2 cannot be tested directly because the enzyme is of human origin. However, research in several labs on whole body distribution using radioactive MC (four differently labeled isotopes) indicates that brain uptake must be very low. Nevertheless, this issue and the possibility of equivalent transporters in the rat and mouse should be examined. As a first step, mammalian/human cells transfected with the OATP1A2, or any other cell that expresses the transporter, should be tested for MC uptake and intracellular changes such as protein phosphatase activity inhibition and cytoskeletal damage. It is also necessary to confirm that cell lines expressing those OATPs as found in liver can take up MC with activity similar to that found in hepatocytes.

Cylindrospermopsin. It is thought that CYN permeates membranes in all cells, although further experimental demonstration is needed. This can be accomplished only when radiolabeled CYN becomes available. There is some indication that hepatocytes are more sensitive to CYN toxicity than other cells. This could be due to differences in metabolism and/or uptake.

Research Priority: Determine the role of metabolism in toxicity and detoxification

The accepted pathway for microcystin excretion is conjugation to the thiol of glutathione (GSH). This may be excreted as MC-GSH or processed to the gamma glutamyl cysteine conjugate and finally to the cysteine conjugate, and then excreted. These reactions have been shown to occur enzymatically *in vitro* with cell extracts from many sources (i.e., Pflugmacher

et al. 1998, Pflugmacher et al. 2001, Takenaka 2001). This is the most likely *in vivo* pathway given the changes in GSH peroxidase and in GSH transferases that follow MC dosing. Evidence of this pathway in intact cells or *in vivo* after MC dosing includes detection and identification of MC metabolites formed *in vivo* in mouse and rat liver (Kondo et al. 1992, Kondo et al. 1996). The GSH adduct (through nucleophilic reaction of the thiol group of GSH to the alpha-beta unsaturated carbonyl of the Mdha moiety (methyl dehydroalanine)) was formed as a small (not specified) percentage of the dose in mice injected i.p. with MC-RR (LD_{50}) between 3 and 24 hr. Sipia et al. (2002) and Karlson et al (2003) reported the presence of the GSH conjugate of nodularin as well as native nodularin in mussels and flounders off the Finnish coast. A nodularin conjugate has also been found in *Artemia salina* (Beattie et al. 2003). Wiegand and Pflugmacher (2005) reported that MC-GSH has a lower affinity for protein phosphatase than the native MC. Although MC excretion in mammals has been proposed to be mainly intestinal, the chemical nature of excreted MC has not been determined. Experimental studies are needed to determine and quantify the metabolism and excretion of MC and metabolites.

There is evidence for metabolic activation of cylindrospermopsin: inhibitors of CYP450s reduce CYN acute toxicity (Runnegar et al. 1994, Froscio et al. 2003), genotoxicity (Humpage et al. 2005), and CYN-dependent inhibition of glutathione synthesis (Runnegar et al. 1995). As noted above, Norris et al. (2001 and 2002) found evidence of CYN/or metabolite binding to protein in the liver, and HPLC analysis indicated that this tightly bound CYN derivative was in part a hydrophilic metabolite with a different elution time. For both MC-LR (and congeners) and CYN, research is needed to characterize the formation, chemical nature, and toxicity of metabolites in animals and humans.

Exposure experiments indicated that fish are susceptible to saxitoxins when administered orally or by i.p. injections, and cytochrome P4501A was induced, suggesting a role for the enzyme in saxitoxin metabolism (Gubbins et al. 2000). Saxitoxin composition and concentrations in mussels and human biological specimens demonstrated that toxin metabolism in humans occurs. Following a human fatality associated with consumption of crabs contaminated with saxitoxins, toxin compositions in the crab and clinical samples (human gut, liver and urine) indicated that toxin metabolism occurred and that toxin conversion began in the victim's gut (Llewellyn et al. 2002).

Charge 2: Identify and prioritize toxicokinetic research needed to improve human health risk assessments.

The recent Harmful Algal Research and Response plan (HARRNESS 2005) indicates top priorities for biosynthesis and metabolism research include: 1) identifying metabolites that contribute to animal or human illness; 2) identifying metabolites that are useful as biomarkers for longer term exposure and; 3) providing toxicological and pharmacokinetic information on HAB toxins and metabolites to help determine toxin uptake, metabolism, and clearance rates. We concur with these research needs and further delineate research priorities as follows:

Near-term Research Priorities

- Provide labeled compounds and better characterized antibodies for toxicokinetic studies
- Develop standardized methods for sample preparation for analysis of toxins/metabolites in complex matrices (tissues)
- Classical toxicokinetics (distribution, half-life, elimination, etc.) in laboratory animals

Long-term Research Priorities

- Determine role of metabolism (if any) in toxicity and detoxification
  - Identify metabolites for priority toxins
  - Mixtures issues: do toxins share common metabolic pathways; are enzymes induced or inhibited that could affect toxicity of other toxins or repeated exposures?
  - Is the promotion of oxidative stress an additional toxic factor of cyanobacterial toxins?
  - Human *in vitro* metabolism (tissue, cell and enzyme specific studies)
- Apply toxicokinetic methods to describe exposure route-dependent and species-specific differences in toxicity
  - Develop physiologically-based toxicokinetic models for priority toxins
- Developmental toxicokinetics
  - Describe transport of priority toxins across placenta
  - Describe the toxicokinetics of saxitoxin in developmental neurotoxicity

Charge 3: Toxicodynamics

Identify and prioritize toxicodynamic research needed to improve human health risk assessments.

Research Priority: Assess dose-response relationships and mechanisms with a focus on low concentration exposures including both *in vitro* and *in vivo* studies as appropriate

Acute Toxicity and Known Mechanisms

Microcystins. Microcystins cause adverse effects by rapidly binding and inhibiting the activity of ser/thr protein phosphatases PP1 and PP2A. The inhibition is irreversible and its consequences are well characterized for acute toxic doses (Ito et al. 2002). MC-LR has a LD_{50} (i.p., mice, 24hr) of 60 μg/kg. The primary acute effect of protein phosphatase inhibition is hyperphosphorylation of many cellular proteins including the hepatocellular cytoskeleton, which causes loss of cell-cell contacts and thus intra-hepatic hemorrhage. Death is due to hypovolemic shock (Runnegar and Falconer 1986; Falconer and Yeung 1992; Runnegar et al. 1993). Other acute effects include altered mitochondrial membrane permeability, generation of reactive oxygen species, and induction of apoptosis (Fladmark et al. 1999; Humpage and Falconer 1999; Ding et al. 2000; Hooser 2000), likely due to a fatal loss of control of regulatory phosphorylation. Morphological changes have been characterized using histopathologic and electron microscopic techniques in rodents, pigs, sheep, and chickens. The Caruaru hemodialysis incident in Brazil extended these findings to humans (see Jochimsen et al 1998; Pouria et al. 1998; Azevedo et al. 2002; Soares et al. 2006; Yuan et al. 2006).

Bulera et al. (2001) showed by toxicogenomic analysis that a non-lethal i.p. injection of MC-LR in male rats resulted in a distinctive pattern of gene expression change when probed by microarray with 1600 rat genes at 3 and 6 hrs after dosing. The pattern differed from that seen with other well characterized liver toxins. The dose of MC (50 μg/kg) did not cause any detectable histological change. This is consistent with the changes in liver function such as receptor-mediated endocytosis that is seen in hepatocytes at doses of MC-LR that cause no significant cytoskeletal changes (Hamm-Alvarez et al. 1996; Runnegar et al. 1997). Although MCs may affect cellular activities independent of the phosphatase inhibition, the impacts of protein phosphatase inhibition may directly or indirectly account for hepatocyte functional alterations, oxidative stress, apoptosis, and

changes in gene expression. Protein phosphorylation status plays a critical role in cellular signaling and hence all aspects of cellular function. In addition a number of these effects are also found in cells treated with other chemically distinct protein phosphatase inhibitors such as okadaic acid (see Gehringer 2004 for a review).

Protein phosphatase inhibition by MC is mediated principally by the ADDA group (Goldberg et al. 1995), although most microcystin variants contain dehydroalanine which can covalently link to a cysteinyl sulphur on the phosphatase (Humpage this volume). Congeners of MC-LR with an intact ADDA will also inhibit protein phosphatases. The covalent binding of N-methyl dehydroalanine to cys is secondary in protein phosphorylation, but is important in the metabolism of MC. GSH competes for binding at the N-methyl dehydroalanine to form the GSH conjugate of MC.

Cylindrospermopsin – cytotoxicity. LD_{50} experiments with CYN indicate a delayed toxicity (2.0 mg/kg, i.p. mouse, after 24 hrs but 0.2 mg/kg after 5 days; Ohtani et al. 1992). The pathology caused by CYN has been studied by light and electron microscopy in male mice dosed i.p. or orally. The liver and kidney were the organs most affected (Falconer et al.1999). Acute CYN poisoning results in lipid accumulation in the liver followed by hepatocellular necrosis (Terao et al. 1994; Seawright et al. 1999). Non-hepatic effects include destruction of the proximal tubules of the kidney (Falconer et al. 1999), as well as cytotoxic and thrombotic effects in other tissues. In addition, CYN extract caused varying lesions in other organs as reported earlier (Hawkins et al. 1985). Sub-chronic oral exposure resulted in mainly hepatic and renal effects (Humpage and Falconer 2003). Effects of poisoning in humans included hepatoenteritis and renal insufficiency (Byth 1980).

Native CYN has been shown to be a potent and irreversible inhibitor of protein synthesis at the translation step (Froscio et al. 2001; Runnegar et al. 2002; Froscio et al. 2003). It has also been shown to inhibit GSH synthesis (Runnegar et al. 1995b). However, cell death and the pathology in CYN-dosed animals cannot be explained fully by protein and GSH synthesis inhibition. Further, there is protection from CYN toxicity by cytochrome P450 inhibitors (Runnegar et al. 1995b; Froscio et al. 2003). The best interpretation is that a CYN-derived metabolite formed by a P450-catalyzed reaction is responsible for increased and/or different toxicity compared to that caused by the parent CYN. This is in agreement with the finding that the LD_{50} is lower (more toxicity) at 7 days than after one day in mice (Hawkins et al. 1985; Ohtani et al. 1992; Froscio et al. 2003). Perhaps the early toxicity is due to P450-dependent activation, and the later

toxicity is the result of protein synthesis inhibition or a combination of the two effects.

Chemical intermediates in the synthesis of CYN showed that the sulfate ester is not necessary for toxicity since the CYN-DIOL (lacking the sulfate) inhibited protein synthesis in hepatocytes with the same potency of native CYN. The orientation of the C-7 hydroxyl did not matter as the epimer was toxic with similar potency (Runnegar et al. 2002). More recently it was found that synthetic deoxy-CYN (lacking the hydroxyl group at C-7), like natural deoxy-CYN, retains the toxicity of CYN (Looper et al. 2005). Treatment of CYN with chlorine yielded 5-chloro-CYN and cylindrospermic acid. These degradation compounds were shown to have at least 50-fold less toxicity than intact CYN in a mouse bioassay (Banker et al. 2001).

In contrast to MC, more work needs to be done to determine the mechanism of toxicity of CYN both in vitro and in vivo. At present, neither the structural determinants of CYN toxicity nor the cellular targets of CYN have been elucidated. Effects of cylindrospermopsin on non-hepatic tissues have not been well investigated. Renal effects seemed to be important after chronic low dose exposure (Humpage and Falconer 2003), and thrombotic effects have been described by a number of researchers. Preliminary results from reproductive and teratogenic toxicity assessments indicate that larger studies will be required.

Cylindrospermopsin – genotoxicity. Cylindrospermopsin has been shown to be genotoxic in various *in vitro* assay systems. Concentrations of 1 – 10 μg/ml (2.4 – 24 μM CYN) caused DNA fragmentation and loss of whole chromosomes in the cytokenesis-blocked micronucleus assay using the human white blood cell-line WIL2-NS (Humpage et al. 2000a). However, CHO K1 cells did not show this effect when exposed to 1.2 – 2.4 μM CYN (Fessard and Bernard 2003). To investigate the role of metabolism in this discrepancy, genotoxicity was studied in primary mouse hepatocytes (Humpage et al. 2005). Cylindrospermopsin-induced DNA fragmentation, as detected by the COMET assay, was seen at CYN concentrations as low as 0.05 μM, well below the cytotoxicity EC_{50} of 0.5 μM. Genotoxicity was eliminated by application of the CYP450 inhibitors omeprazole (100 μM) or SKF525A (50 μM) (Humpage et al. 2005), indicating that a CYN metabolite probably induces DNA fragmentation. More recently, a range of standard *in vitro* assays have been used to confirm that DNA fragmentation is the major mechanism leading to genotoxicity (Humpage this volume). Research is needed to identify metabolic pathways and toxic metabolites that produce acute toxicity and genotoxicity. There is considerable evidence for involvement of CYP450s, but the specific isoforms

have not been identified. Isoform identification is needed to determine whether toxic biotransformation pathways occur in humans and to assess the applicability of animal data to human risk.

In vivo, DNA fragmentation was described in the livers of mice treated with cylindrospermopsin (Shen et al. 2002). Intra-peritoneal injection of a single dose of 0.2 mg CYN/kg induced an increase in hepatic DNA strand breakage within 6 hours that normalized by 72 hours. Evidence from ^{32}P-post-labeling experiments using the livers of CYN-treated mice also suggested that DNA adducts are formed (Shaw et al. 2000).

Saxitoxins. The saxitoxins are potent voltage-gated sodium channel antagonists, causing numbness, paralysis and death by respiratory arrest. Analogue potency varies greatly, with saxitoxin having an LD_{50} (i.p. mouse) of 10 µg/kg, but saxitoxin C1 being at least 160-fold less toxic (Oshima 1995). Toxicological studies to date have assessed acute exposure effects, as would be expected from shellfish poisoning. Repeated, low-level exposure studies are needed to assess effects from drinking water exposure (Humpage this volume). Research is needed to verify and extend a report that tolerance to PSPs develops (Kuiper-Goodman et al. 1999). Mammalian studies are needed to extend a demonstration of neuro-developmental disturbances in fish (Lefebvre 2002).

Toxicodynamic studies have been performed for gonyautoxins (GTXs) in cats to assess their toxic effects and intoxication illness in mammals when administered orally (Andrinolo et al. 2002). The primary physiological effect was a dramatic decrease in arterial pressure, which eventually resulted in death. Oral doses of 35 µg/kg of GTX 2/3 epimers and a plasma level of 36 ng/ml were the lethal limits for cats. The specific binding of saxitoxin to brain tissue of mammals (Trainer and Baden 1999; Llewellyn et al. 2004; Cianca et al. 2007) and sub-lethal, short term exposure of dissolved saxitoxin to larval fish (Lefebvre et al. 2004) have also been examined. In rats dosed i.p. with 5 or 10 µg saxitoxin/kg body wt, saxitoxin was bound to sodium channels in all brain regions at ppm levels. Larval fish exposed to dissolved saxitoxin exhibited reductions in sensory-motor function after 48 h and paralysis by 4 days. Larval exposure also resulted in reduced growth and survival of the fish several weeks later.

Nodularins. Nodularins are hepatotoxic cyclic peptides of similar structure to the microcystins, containing 5 amino acids rather than 7 (Rinehart and Namikoshi 1994). ADDA is still present but dehydroalanine is replaced by N-methyl-dehydrobutyrine (Rinehart et al. 1988). The smaller ring size prevents this latter moiety from coordinating with the phosphatase cysteine, preventing nodularin from bind covalently (Lanaras et al. 1991;

Craig et al. 1996; Bagu et al. 1997). However, due to the high affinity of ADDA for the active site, this lack of covalent binding does not affect toxin potency, which is similar to that of microcystin-LR (Ki's are of the order 0.1 – 1.5 nM; (Honkanen et al. 1990; MacKintosh et al. 1990; Honkanen et al. 1991)). The lack of covalent binding may allow nodularin to reach other sites in the cell, a mechanism by which nodularins may act as direct carcinogens (Ohta et al. 1994; Bagu et al. 1997; Humpage this volume).

Anatoxin-a/Homoanatoxin-a. These toxins are nicotinic acetylcholine receptor agonists having a LD_{50} of 200 μg/kg (Carmichael et al. 1979; Carmichael 1994). Residence of these toxins at post-synaptic cholinergic receptors results in nerve depolarization (Swanson et al. 1990; Huby et al. 1991; Swanson et al. 1991; Wonnacott et al. 1991). Typical symptoms in mice are loss of muscle coordination, gasping, convulsions, and death within minutes from respiratory arrest (Carmichael et al. 1979). Dog deaths have been attributed to poisoning by these toxins when the animals have licked their coats after swimming (Codd et al. 1992; Edwards et al. 1992; Falconer and Nicholson, pers. comm.). A single human fatality was attributed to anatoxin-a poisoning by a county coroner after the victim swam in a scum-covered pond (Behm 2003). Although anabaena was identified in stool from the victim and another swimmer who became ill but survived, mass spectrometric analyses for anatoxin-a in blood were inconclusive (Carmichael, pers. comm.). The peaks for anatoxin-a and the indigenous amino acid, phenylalanine, occur in the same portion of the spectrum because these compounds are isobaric and elute similarly in reversed phase liquid chromatography (Furey et al. 2005). Anatoxins have not been linked to human poisoning via drinking water (Humpage this volume).

Anatoxin-a(s). Anatoxin-a(s) is a phosphorylated cyclic N-hydroxyguanine, with a structure and action similar to organophosphate pesticides (Mahmood and Carmichael 1986, 1987; Hyde and Carmichael 1991). It is a potent acetylcholinesterase inhibitor with a LD_{50} (i.p., mouse) of 20 μg/kg. The *in vivo* toxic effects are similar to those of anatoxin-a but with the addition of salivation (hence the "s") and lacrimation (Mahmood and Carmichael 1986, Carmichael 1987, Matsunaga et al. 1989). No human illness has been attributed to this toxin (Humpage this volume).

Research Priority: *In vitro* and *in vivo* studies of chronic and repeated exposures

Subchronic and Chronic Toxicity

Microcystins. Reliable *in vitro* data are lacking because hepatocytes lose the transporters for MC with time in culture; thus, repeated/continuous exposure is not possible. This problem can now be overcome in part by using other cell lines that have been transfected to express the newly identified transporters. The effects of low doses of MC have been studied over a period of 65 hrs (Humpage and Falconer 1999). The authors reported a dose dependent (10-100 pM) balance between proliferation and apoptosis of hepatocytes in culture. Lower microcystin doses appeared to affect cell cycle control by suppressing apoptosis and promoting cell division in polyploid hepatocytes *in vitro* (Humpage and Falconer, 1999; Lankoff et al. 2003). Microcystin has also been shown to inhibit DNA repair (Lankoff et al. 2004; 2006a,b). These effects are consistent with the tumor promotion seen in *in vivo* studies, such as enhancement of the growth of hepatic and colonic pre-cancerous lesions in animal models (Fujiki and Suganuma 1993; Ito et al. 1997a; Humpage et al. 2000b; Sueoka et al. 1997). Microcystin exposure has been linked to human liver and colon cancer incidence (Yu 1995; Fleming et al. 2002; Zhou et al. 2002). Using an intestinal crypt cell line, Zhu et al. (2005) explored the mechanism of tumor promotion that is shared by MC and chemically unrelated protein phosphatase inhibitors such as okadaic acid, and showed that MC transformed these cells. Transformation is an important step in the development of carcinogenesis. These cells also had increased proliferation, and the authors went on to show that MC treatment resulted in increased expression and activity of proteins that control cell cycle signaling such as Akt, JNK and MAPK pathways. This study also provides a mechanistic explanation for the hyperplasia in the colon seen in mice initiated with azoxymethane and then exposed to MC in the drinking water for 212 days (Humpage et al. 2000b). To date, findings for MC do not clearly and unequivocally demonstrate a potential for carcinogenicity. The substantial evidence of tumor promotion by MC has recently led the Agency for Research on Cancer (AIRC) to classify MC-LR as "possibly carcinogenic to humans" (Group 2B). A summary of this evaluation has been published (Grosse et al. 2006), and a full report will be published as an AIRC monograph.

A number of rodent studies (and one pig study) with repeated dosing of MC or bloom extracts containing MC have been reported. Some of these studies have been used to calculate reference doses (tolerable daily intakes), LOAELs and NOAELs. These will be addressed by other groups.

We have some indication of the effect of repeated sublethal dosing: daily i.p. dosing with MC-YM for up to 28 days resulted in mild liver damage (Elleman et al. 1978). Bloom extract given over 1 year orally to mice caused liver changes at the higher doses and some indication of increased incidence of tumors (Falconer et al. 1988). Oral consumption of MC containing cells by pigs for 8 weeks resulted in dose dependent liver toxicity (Falconer et al.1994). Miniosmotic pump continuous administration of MC-LR in male rats for 28 days resulted in dose dependent increases in hepatic oxidative stress, in several serum biochemical toxicity indicators, and in histopathology that correlated with protein phosphatase inhibition (Solter et al. 1998; Guzman and Solter 1999). Oral and i.p. dosing of mice with MYC-LR caused dose-dependent liver lesions, but no evidence of developmental toxicity in pregnant females (Fawell et al. 1999). No toxicity was observed in female mice dosed orally with 20 µg/L of MC-LR over 18 months (Ueno et al. 1999).

Cylindrospermopsin. Oral toxicity of low levels of CYN have been investigated by Humpage and Falconer (2003) in mice over 10-11 weeks. Dosing with a *Cylindrospermopsis* culture extract and with CYN indicated that toxic changes were dose dependent with the kidney being most sensitive to the toxin. In addition, a study by Falconer and Humpage (2001) showed the potential for carcinogenicity of CYN-containing culture extracts. Male mice were observed for 30 weeks after three sublethal doses of *C. raciborskii* extract were given orally over a 6 week period followed by treatment with TPA. At the dose used (equivalent of 2.75 to 8.25 mg CYN), the mice exhibited none of the histological changes seen in acute CYN toxicity. However, this treatment appeared to induce the formation of overt cancers in the mice by 30 weeks. Five of 53 treated mice developed neoplastic lesions whereas none of 27 controls did so. The odds ratio for carcinogenesis following CYN treatment was 6.2, but the 95% confidence interval on this estimate was 0.33 to 117, a statistically insignificant result ($p=0.16$). Nevertheless, this study presents the clearest evidence yet that cylindrospermopsin is not only genotoxic but may also be carcinogenic, which has implications for low-level repeated exposures to CYN. The potential carcinogenicity of CYN needs to be investigated in a larger study. Because this study used *Cylindrospermopsis* culture material, it is not possible to state that CYN alone was the causative agent. The potential carcinogenicity of CYN and of CYN containing extracts should be investigated in a larger number of animals, as well as in a non-rodent species. Cylindrospermopsin has been placed on the 'candidate list' for full toxicological studies by the USEPA and NIEHS. This can only be accomplished if large

amounts of CYN and CYN containing extracts are prepared and made available.

Research Priority: Cyanotoxin Mixtures

An important point to reiterate is that single microcystins or single cylindrospermopsins almost never occur in nature. Instead mixtures of toxins are the norm, so there is a need to understand toxin interactions. This will enable the calibration of studies done using MC-LR and the formulation of regulations to be based on toxicity equivalents rather than quantities of individual compounds.

Crude extracts from blooms have been found to be more toxic than the purified component toxin(s). This is probably due to the presence of other congeners or classes of toxins that enhance toxicity. Similar patterns appear in many studies of chemical ecology (Paul et al. 2001). Other compounds present in minor concentrations may have additive or synergistic effects with the pure compounds of interest. A research need is to characterize the compounds in crude extracts that enhance the toxicity of pure compounds, and to identify whether any compounds in crude extracts can reduce the toxicity of pure compounds. An additional research need is to characterize interactions between cyanotoxins and other bioactive compounds in raw and finished water such as metals or disinfection byproducts.

Charge 3: Identify and prioritize toxicodynamic research needed to improve human health risk assessments.

General

- Determine effects from low concentration exposures starting with *in vitro* studies, proceeding to *in vivo* studies as appropriate.
- In contrast to MC, more work needs to be done to determine the mechanism of toxicity of CYN both *in vitro* and *in vivo,* including the structural determinants of CYN toxicity and the cellular targets of CYN.
- Studies are needed to assess the effects of repeated and chronic exposures to individual cyanotoxins and appropriate mixtures.

Mixtures

Near-term Research Priorities

- Mixtures of toxins are the norm, so we need to understand toxin interactions.
  - Priorities are mixtures of microcystins and cylindrospermopsin
  - Use *a priori* information (regional occurrences) to prioritize candidate mixtures

Long-term Research Priorities

- Congener mixtures, especially effects of mixtures of microcystin analogues
- Determine and characterize compounds in the crude extracts that enhance the toxicity of pure compounds, and identify whether any compounds in crude extracts can reduce the toxicity of pure compounds.

Individual Toxins

- *Microcystins: Near-term Research Priorities*
  - Analog mechanisms (including non-toxic ones) in comparison to MCY-LR
  - Comprehensive studies of teratogenicity and reproductive toxicity
  - Chronic animal studies to assess carcinogenicity (ideally these studies should test cyanobacterial extract that may contain modifiers of toxicity (as would be present in water) and with purified MC

- *Microcystins: Long-term Research Priorities*
  - Epidemiological studies into links with human cancer. This requires a biomarker of low dose exposure for which ELISA may be an option (Hilborn et al. 2005).
  - Long term repeated dosing studies are needed in species other than rodents
  - Evaluate effects of modified MCs (and other cyantoxins) produced as byproducts of water treatment
- *Cylindrospermopsin: Near-term Research Priorities*
  - Genotoxicity mechanism including the role of specific CYP450 isoforms in CYN activation and identification of genotoxic metabolites
  - Identification of biomarkers of exposure and effect
  - *In vivo* carcinogenicity trials when adequate pure toxin is available

- Detailed toxicokinetics, including distribution and binding studies are needed to explain the observed delayed toxicity
- Studies of extra-hepatic effects including renal, thrombotic, immunologic, reproductive, and developmental effects
- Human effects – An opportunity exists for follow-up of exposed humans on Palm Island, Australia

- *Anatoxins and Saxitoxins*
  - Chronic low dose exposures/neural developmental toxicity mechanisms *(near-term research priority)*
  - ATXa(s) – studies comparing ATXa(s) and organophosphate pesticide toxicity *(longer-term research priority)*

- *BMAA: Near-term Research Priority*
  - Further assess the association with neurodegenerative disease (Cox and Sacks 2002)

- *BMAA: Long-term Research Priorities*
  - Mechanism of bioaccumulation.
  - Mechanism of toxicity (glutaminergic excitotoxicity or other effects such as disruption of protein structure/function).
  - Trophic studies to determine routes of human exposure.

- *Lower priority*
  - Nodularins – use microcystin as model
  - Lyngbya toxins

Charge 4: Susceptibility

> Identify and prioritize research needed to improve our understanding of human susceptibility factors for adverse effects from cyanotoxin exposure.

Factors affecting susceptibility to cyanotoxins have generally not been investigated for the majority of the toxins. The elucidation of toxic mechanisms as described above within the toxicokinetics and toxicodynamics charges will provide direction and insights for research on susceptibility. Comparative toxicology studies of microcystins have yielded some findings relevant to susceptibility, as summarized below.

In vivo Differences in Responses to Microcystins

There is evidence that nutritional status, sex, age, and strain of laboratory animals can influence the severity of *in vivo* MC toxicity. Mechanistic explanations for these variations in susceptibility are unknown. Fasted rats were more sensitive to MC toxicity (Miura et al. 1991). The LD_{50} for fasted rats dosed i.p. was half that of fed rats (72 μg/kg and 122 μg/kg, respectively). Several studies have indicated that male mice are more sensitive than females to the effects of repeated doses of MC (Falconer et al. 1988). Age can also influence susceptibility to MC; older male mice were more sensitive to oral doses of MC-LR than young male mice (Ito et al. 1997b). Strain differences were demonstrated when Ito et al. (2000) found that Balb/C mice were more sensitive to MC-LR than ICR mice following 12 weeks of repeated oral dosing. It is reasonable to expect that differences in sensitivity to MCs will also be present in human populations.

Researchers have also compared MC toxicity resulting from different dosing modalities. Liver damage from ileal loop dosing was similar to that observed after intravenous dosing (Stotts et al. 1997a, 1997b; Dahlem et al. 1989). Intracheal administration of MC-LR to male mice was as toxic as i.p. doses, with a delay of 60 min (Ito et al. 2001). Inhalation exposure of BALB/C mice to 260–265 mg MC-LR/m3 for 0.5, 1.0, or 2.0 hours/day for 7 days resulted in degeneration and necrosis of the respiratory epithelium but no liver damage (Benson et al Toxicon 45: 691-698, 2005). The lack of hepatotoxicity was attributed to the deposited dose (2.5 mg microcystin/kg in the high-dose group) being far below the no observable adverse effect level (NOAEL) of 200 mg/kg/day for induction of hepatotoxicity.

Charge 4: Identify and prioritize research needed to improve our understanding of human susceptibility factors for adverse effects from cyanotoxin exposure.

The Harmful Algal Research and Response plan (HARRNESS 2005) also prioritized studies to investigate integrated toxin effects and mechanisms of susceptibility with emphasis on characterizing acute and long term effects of HAB toxins, defining mechanisms of susceptibility (e.g. identifying special risk populations), and integrating laboratory animal model data and wildlife exposure information with human exposures and disease. We concur with these research needs and further delineate research priorities below.

Near-term Research Priorities

- Mechanisms as indicated by toxicokinetics/toxicodynamics work
  - P450/GST profile–susceptibility may be determined by polymorphically expressed metabolizing enzymes
    - Focus on links between CYN metabolism by CYP450s and human susceptibility due to variations in expression of human CYP enzymes
    - MC susceptibility may be similarly affected by variability in GSTs

Long-term Research Priorities

- Race, age, sex, and individual health status
- Nutritional status
- Epidemiological studies with an emphasis on dose/exposure characterization
- Identification of special risk populations through integration of laboratory animal model data and wildlife exposure information with human exposures and disease

Charge 5: Genetics/OMICS of Cyanobacterial Toxin Production

Identify and prioritize genetic/OMICS research needed to improve the prediction of cyanobacterial HABs.

Research Priority: Characterization of genomes, transcriptomes, and proteomes

Whole genome sequencing of *Microcystis, Cylindrospermopsis, Lyngbya,* and *Anabaena* and bioinformatic analyses are of high priority and may be more cost-effective than attempting to isolate individual genes that influence the expression of the toxin gene clusters. Whole genome sequencing produces huge amounts of data in a short time and enables the characterization of biosynthetic pathways and the development of tools, such as microarrays, needed to describe synthesis regulation. As sequencing costs are constantly declining this option is becoming more and more feasible for most projects. The Sivonen lab in Helsinki, Finland, is nearing

completion of the whole genome sequencing of a hepatotoxin-producing *Anabaena*, and a microcystin-producing *Microcystis* genome is nearly finished at the Pasteur Institute. However, the present capacity and speed of sequencing in USA-based institutes is such that more genomes can be done relatively quickly and cheaply. Determining more whole genome sequences from these harmful cyanobacteria will allow comparisons between, for example, toxic and non-toxic genomes of the same species, speeding up identification of not only toxin genes but also regulatory genes involved in toxin expression. We propose that priority should be given to determining the whole genomes of a second *Microcystis*, toxic and non-toxic strains of both *Cylindrospermopsis* and *Lyngbya*, and two neurotoxic *Anabaena* strains – one saxitoxin producer and one anatoxin-a producer. If whole-genome sequencing is not possible, the toxin gene clusters should at least be identified, thereby enabling the development of PCR-based assays for potential toxin production. In addition to whole genome sequences, global transcriptional and proteomic analyses would reveal mRNA transcripts and proteins correlated with the expression of cyanobacterial toxins. This information would advance our understanding of how toxin production is regulated by cyanobacteria and in response to the ecophysiologic influences of external forces (e.g., light, nutrient availability, etc.).

The complete biosynthetic gene clusters are known for microcystin and nodularin. This information has allowed for detailed studies on the regulation of cyanotoxin production and the development of molecular tools for detecting particularly harmful species in the environment. These genes can be used as an indicator of potential toxin production, alerting water quality managers to the possibility of a toxic bloom. Reservoirs can be monitored by extracting DNA from whole algal communities, and using a PCR-based assay to test for the presence of a gene fragment involved in coding the microcystin synthetase complex (e.g., Hisbergues et al. 2003). Genetic information is also critical for detecting and understanding the production of other cyanobacterial toxins. Only a partial gene cluster analysis has been derived for cylindrospermopsin synthetase. Identification of the complete genetic sequence for cylindrospermopsin synthetase should be given high priority due to the prevalence and distribution of this toxin. Of lower priority are the gene clusters encoding the synthesis of the anatoxins, saxitoxins, and other paralytic shellfish poisons found in freshwater. It is proposed that a strain of C. raciborskii AWT205 (cylindrospermopsin), Oscillatoria or Anabaena spp. (anatoxin-a and anatoxin-a(s)), and either C. raciborskii T3 or a strain of A. circinalis (saxitoxin) be chosen for genome sequencing. Contaminating bacteria in co-culture with these cyanobacteria should be removed prior to DNA extraction and clon-

ing. In addition, genes that interact with the toxin gene clusters remain to be fully described in *Microcystis*, and are completely unknown for the other organisms. The genomic data are needed to apply proteomic and transcriptomic analyses. Global transcriptional and proteomic analyses can reveal mRNA transcripts and proteins correlated with the expression of cyanobacterial toxins.

If whole genome sequencing is not feasible, an alternative approach is to:

- Use global transcriptional and proteomic analyses to locate mRNA transcripts and proteins correlated with the expression of cyanobacterial toxins;
- Use these data to identify the biosynthetic pathways;
- Mutate the pathways to confirm;
- Identify biosynthetic genes for expression;
- Use this information to develop genetic markers for toxin production;
- Communicate: outreach/education to distribute these tools to local water quality agencies.

Research Priority: Identification, characterization, and confirmation of unknown genes for toxin pathways

As noted above, the major cyanobacterial toxin pathways that have not yet been identified or fully characterized are cylindrospermopsin, saxitoxin, anatoxin-a, and anatoxin-a(s). The proposal of a putative biosynthetic pathway has been a successful approach to identifying toxin gene clusters. The first step usually involves feeding experiments with isotope-labeled precursors for the identification of metabolic intermediates. Once these intermediates have been identified, candidate enzymes can be postulated to catalyze these reactions. The next step usually involves searching for genes that encode those putative enzymes in the producing organisms, via degenerate PCRs targeting of conserved domains in these enzyme families, or by screening genomic libraries with labeled probes for putative genes. The most suitable target genes encode unique enzymes that are not involved in primary metabolic pathways. Because cyanobacterial (and other bacterial) toxin genes are organized into clusters, it is usual that the rest of the enzymes involved in the biosynthesis are located adjacent to the identified candidate gene. Following the identification and sequencing of a candidate gene, "genome walking" techniques such as adaptor-mediated and inverse

PCR can be implemented in order to sequence regions around the candidate gene, thereby revealing whether this candidate gene is part of the entire toxin gene cluster.

Confirmation of the role of a putative toxin gene cluster in toxin production is usually accomplished by creating a mutant strain. Confirmation is attained when deletion of a critical part of the toxin gene cluster abolishes toxin production. Mutation methods include gene deletion, insertional mutagenesis, and point mutation. Candidate organisms for anatoxin-a toxin gene cluster identification and confirmation include *Anabaena flos-aquae*, *Oscillatoria* sp., or *Aphanizomenon* sp. The only known producer of anatoxin-a(s) is an *Anabaena* sp. Knockout confirmation of a saxitoxin putative gene cluster could be conducted in a variety of organisms, including *Anabaena, Cylindrospermopsis*, certain heterotrophic bacteria, and several dinoflagellate species. The putative Cylindrospermopsin gene cluster should be mutated in *Cylindrospermopsis raciborskii* or *Anabaena bergii*. *Nodularia spumigena* is suitable for identification and confirmation of a nodularin synthetase gene.

Research Priority: Expression of biosynthetic genes

Cyanobacteria are notoriously difficult to culture in the absence of contaminating bacteria, and methods for genetic manipulation have been described for only a few species. Therefore, verification of putative toxin biosynthesis genes in cyanobacteria by mutational studies is intrinsically difficult. The only cyanobacterial toxin gene cluster that has been characterized by gene disruption and mutant analysis is that for microcystin in *Microcystis*, *Anabaena*, and *Planktothrix*. An alternative approach to gene disruption and mutant analysis is the cloning, heterologous expression, and biochemical characterization of purified recombinant protein. Several cyanobacterial toxin gene clusters have been identified by screening genomic libraries with probes that target candidate biosynthesis genes. Upon identification of candidate gene clusters, the sub-cloning and heterologous expression of candidate genes has led to the characterization of gene clusters for several cyanobacterial metabolites, such as jamaicamide, lyngbyatoxin A, and barbamide. Heterologous expression is not limited to individual genes. Entire gene clusters may also be transferred to metabolically engineered strains that are capable of complex secondary metabolite production, such as *Escherichia coli* BAP1. This approach was successfully used to express the biosynthesis of the siderophore yersiniabactin and a precursor to the antibiotic erythromycin. Once heterologous expression is established in a host strain, which is amenable to genetic manipulation,

mutant analysis can be performed with relative ease. In addition, such a system would be essential for the rational design of metabolic products with enhanced pharmacological activities via combinatorial biosynthesis.

Biosynthetic enzymes in *Cylindrospermopsis raciborskii* T3 have been assayed and there was evidence to indicate differences between the metabolism of STX and the C1+2 toxins and high turnover rates of STX biosynthetic enzymes (Pomati et al. 2004). Saxitoxin metabolic pathways have been studied through examinations of the purine degradation pathway (Pomati et al. 2001).

Research Priority: Gene probe development for strain detection and potential for gene transfer

The development and validation of gene probes for phylogenetic classification and toxin-production potential are needed. Further research is required to validate the currently available toxin gene probes for use on a variety of sample matrices such as water and sediment. Gene probes for paralytic shellfish poisons and the anatoxins have not been developed. Probes are also needed for differentiating cyanobacterial potential to produce the different congeners of microcystin and the saxitoxin-related molecules. The acquisition of toxin gene clusters by non-toxic species and the recognition of new toxins is a constant threat. Continued monitoring is required, using a combination of genetic, microbiological and toxicological approaches.

Charge 5: Identify and prioritize genetic/OMICS research needed to improve the prediction of cyanobacterial HABs.

Research Priorities

- **Genomes, transcriptomes, and proteomes**
  - *Near-term Research Priorities*
    - High priority: achieving the completed sequence of cylindrospermopsin synthetase due to the prevalence and distribution of this toxin
    - There is broad consensus regarding the high priority of including the toxic freshwater cyanobacteria in future genome sequencing projects, particularly *Microcystis, Cylindrospermopsis, Lyngbya*, and *Anabaena*

- *Long-term Research Priorities*
  - Of lower priority are the gene clusters encoding synthesis of anatoxins and saxitoxin and other paralytic shellfish poisons found in freshwater
  - Apply proteomic and transcriptomic analyses for the understanding of the ecophysiology of cyanotoxin production

- **Identification and characterization of unknown genes for toxin pathways**
  - *Near-term Research Priorities*
    - The major cyanobacterial toxin pathways not yet identified or fully characterized are cylindrospermopsin, saxitoxin, anatoxins-a, and -a(s)
    - Identify metabolic precursors using isotope- or radiolabeled feeding experiments
    - Search for genes that encode putative biosynthetic enzymes in the producing organisms
    - Sequence candidate genes and regions around the gene

- **Mutation of gene clusters to confirm role in toxin production**
  - *Near-term Research Priorities*
    - A saxitoxin putative gene cluster could be mutated in any of the toxin-producing organisms where the toxin gene sequences have been found
    - The putative Cylindrospermopsin gene cluster should be mutated in *Cylindrospermopsis raciborskii* or *Anabaena bergii*
    - The nodularin synthetase gene cluster should be mutated in *Nodularia spumigena*.

- **Expression of biosynthetic genes**
  - *Longer-term Research Priorities*
    - Cloning, heterologous expression, and biochemical characterization of purified recombinant protein
    - Screening genomic libraries with probes that target candidate biosynthesis genes
    - Sub-cloning and heterologous expression of candidate genes to characterize toxin-coding gene clusters
    - Once heterologous expression is established in a host strain amenable to genetic manipulation, mutant analysis can be performed

- **Gene probe development for strain detection and potential for gene transfer**
  - *Near-term Research Priority*
    - Further research is required to validate the toxin gene probes
  - *Long-term Research Priorities*
    - Gene probes directed to both phylogenetic and toxigenic markers are also required for the detection of cyanobacteria that produce paralytic shellfish poisons and the anatoxins
    - Develop a series of probes for differentiating cyanobacterial potential to produce the different congeners of microcystin and the saxitoxins

Charge 6: Predictive Model Development

What additional information is needed to develop predictive models for the production and fate of cyanotoxins?

Physical, chemical, genetic, physiological, and ecological information are all needed to develop models to predict the production of cyanotoxins. At present, the genetic mechanisms that regulate toxin production are mostly uncharacterized, although we do know that toxin production can vary in response to ecological factors, including temperature, light availability, nutrient availability, grazing, and competition (Yin et al. 1997, Kearns and Hunter 2000, Thacker et al. 2005). Mechanistic understanding of the interactions between these modulating factors and the expression of biosynthetic genes and cofactors in the target organisms is needed. The capability to predict the fate of cyanotoxins once produced is dependent on knowledge of toxin transport and reactivity/adsorption with various environmental and water treatment matrices.

Genomic and proteomic analyses would advance our knowledge of the cofactors required for toxin expression, including possible promoters of toxin synthesis. These analyses may also reveal how toxins are transported, stored in the cell, or released to the extracellular environment. The natural roles of many cyanotoxins also remain to be described. Although cyanotoxins are often hypothesized to act as defenses against predation, some herbivores preferentially feed on cyanotoxins (Paul et al. 2001, Camacho and Thacker 2006). The growth of sympatric bacteria and algae can be inhibited by cyanotoxins, but secretions from these potential competitors can also regulate toxin production (Kearns and Hunter 2000).

Research Priority: Characterize environmental, biochemical and trophic factors that regulate toxin gene expression

Previous toxin-regulation studies have yielded disparate results, primarily due to a lack of methodological standardization. Different culture techniques (eg. batch cultures, chemostats, turbidistats, etc.) can have a significant impact on experimental outcomes, as can the different methods used for toxin quantification (eg. toxin versus wet or dry cell weight, cell number, total protein content, chlorophyll content, optical density, etc.). Physiological differences between cyanobacterial strains may also affect experimental results and the interpretation of data. Therefore, when investigating the influence of any given environmental parameter on toxin production, the organism's optimal growth requirements must be taken into account before assigning "high/low" values to experimental variables (eg. light intensity, nutrient concentration etc.).

The characterization of natural and engineered non-toxic mutants may be the key to understanding why cyanobacteria produce secondary metabolites. By comparing and contrasting mutant and wild-type responses to different growth conditions, it may be possible to pinpoint the physiological pathways linked to toxin production. The discovery of complete cyanotoxin biosynthesis-gene clusters (eg. anatoxin, saxitoxin, and cylindrospermopsin), and the development of genetic manipulation techniques, is critical to this area of research. Investigating cyanotoxin production at the genetic level, through methods such as RT-PCR and reporter gene analysis may prove to be more efficient and cost-effective compared to traditional end-product analyses. The positive influence of light on the transcription of microcystin biosynthesis genes has been demonstrated. Similar methods may also be employed to investigate the effects of temperature, stress, nitrogen and phosphorus types and levels, and trace metals in other cyanobacteria following the elucidation of their respective toxin biosynthesis-gene clusters. Ultimately, the provision of genome sequences for the model toxin-producing strains will allow the global analysis of genetic transcription, including the interaction between, and regulation of, both secondary and primary metabolism in these bloom-forming microorganisms.

Research on the regulation of toxin production in cyanobacteria has mainly focused on the transcriptional regulation of toxin genes. However, toxin production may also depend on factors such as the availability of cofactors in the producing cells, as well as the stability and post-translational modification of biosynthetic enzymes. One cofactor essential for all polyketide and non-ribosomal peptide producing enzymes is phosphopantetheine, which acts as a covalent attachment site for activated acyl-

and peptidyl chains. Phosphopantetheine is transferred from coenzyme A to a conserved serine residue of acyl- and peptidyl carrier proteins by phosphopantetheinyltransferase. So far, little effort has been made to characterize cyanobacterial phosphopantetheinyltransferases involved in toxin production. Bacteria have also been shown to affect toxin production by algae. Removal of bacteria from saxitoxin-producing dinoflagellate cultures was shown to reduce their toxicity. Similar effects have been observed with domoic acid-producing diatoms. It is believed that trophic interactions between bacteria and algae may be responsible for this effect on toxin production. Research is needed to describe the constituents and interactions in toxin-production pathways.

Research Priority: Mechanisms of toxin transport

Previous toxin-regulation studies have observed an increase in extracellular microcystins under certain growth conditions. The active export of the toxin, by the ABC transporter McyH, has been hypothesized, but not yet proven. The overexpression, purification and reconstitution of cyanotoxin-associated transporters such as McyH into membrane vesicles may enable us to demonstrate transport *in vitro* with radiolabeled toxins. While bioinformatic and mutational data suggest that McyH is responsible for microcystin transport, it is still unclear whether this enzyme transports the toxin to the extracellular environment or to the thylakoid membranes. Intracellular localization of McyH and other cyanotoxin-associated ABC transporters by *in situ* methods may shed light on this area of research. Techniques for observing the intracellular trafficking of all cyanotoxins, including development of specific toxin and transporter antibodies, as well as cyanobacterial imaging methods, are required.

Research Priority: Determine the ecological/physiological role(s) of cyanotoxins

Without question, toxins provide some functional benefit to cyanobacterial. This statement follows from the observation that toxins, or structurally similar compounds, are produced by most of the surveyed cyanobacterial lineages. Equally compelling is the inference that cyanotoxins may play a critical role in cyanobacterial populations or trophic interactions. What remains in question is what, precisely, is that role. Some cyanobacterial toxins may serve as physiological tools to increase survival in hostile environmental conditions, or to enable the colonization of a novel habitat. Others may play a defensive role and act to prohibit the in-

vasion of other strains or species into an occupied niche, limit the advance of neighboring cells, or discourage grazers and predators. An additional role could be as signaling molecules, mediating cell-cell communication or quorum sensing. It is likely that whatever roles cyanotoxins play, these roles change in accordance with the flux of biotic and abiotic components of the environment.

Characterization of the beneficial roles of toxins in cyanobacterial cells or populations requires delineation of the molecular effects of the specific toxins on the physiology and fitness of the producing microorganisms, populations, and ecology of the various species. Knowledge of the genetic basis for cyanotoxin biosynthesis and regulation will enable a functional genomic approach to the study toxin roles. DNA or protein chips, and gene/protein expression profiling tools in general, could offer valuable data on co-transcription of toxic genes/proteins with other genetic networks in experimental sets chosen to mimic changing environmental conditions. Such conditions could also include the stress induced by other competing microorganisms, predators or grazers. A functional association of toxin biosynthetic genes with cellular and metabolic responses to specific stressors will undoubtedly help in elucidating the toxins molecular role. Protein expression studies could provide further information regarding the activation of metabolic pathways (e.g., phosphorylation of signaling proteins) in association with toxin production, and the functional roles of toxins in activities such as cyanobacterial quorum sensing and perception.

Mutagenesis and gene disruption techniques could confirm functional genomic data, and be crucial for investigating the role of toxins in the physiology of producing cyanobacteria. The function of any given cyanotoxin, as well as the conditions that induce variation in both toxin production levels and toxin structure, could be elucidated by studying toxin gene mutants. Broad physiological parameters (e.g., growth) are needed to understand how mutations in toxic genes affect single-cell phenotypes or the structure of an entire population. Both predators and bacteriophage viruses have been shown to play important roles in the ecology and dynamics of cyanobacterial species. The role of protozoan predation on the natural selection of toxin-producing cyanobacterial species is not clear. To date, possible connections between bacteriophage and the production of cyanobacterial toxins have not been explored. These data are needed to produce theoretical and empirical models of the conditions that favor the proliferation of toxin-producing cyanobacteria.

A clearer understanding of interactions between cyanobacterial physiology and physical, chemical, and biological factors such as light availability, nutrient availability, grazers, and competitors is needed to produce

models that successfully predict the production and fate of cyanotoxins. Knowledge of mechanisms regulating the expression of biosynthetic genes and cofactors in the target organisms is needed to characterize these interactions. Although these studies can be conducted using global transcriptional and proteomic assays, greater sensitivity and power would be gained if the biosynthetic pathways were known a priori. Integrating these studies with knowledge of the entire genome sequence would provide additional predictive power.

Charge 6: What additional information is needed to develop predictive models for the production and fate of cyanotoxins?

Genetic, physiological, and ecological information are all needed to develop a model to predict the production of cyanotoxins.

Research Priorities

- **Environmental effects on toxin production**
  - *Near-term Research Priority*
    - Methodological standardization for toxin regulation studies - take into account organism's optimal growth requirements before assigning "high/low" values to experimental variables (eg., light intensity, nutrient concentration etc.)
  - *Long-term Research Priorities*
    - Characterize natural and engineered non-toxic mutants to understand why cyanobacteria produce secondary metabolites
    - Compare and contrast mutant and wild-type responses to different growth conditions to pinpoint physiological pathways linked to toxin production
    - Investigate cyanotoxin production at the genetic level, through methods such as RT-PCR and reporter gene analysis to examine the effects of light, temperature, stress, nitrogen source and level, and trace metals in cyanobacteria following the elucidation of their respective toxin biosynthesis-gene clusters
- **Other factors affecting toxin production**
  - *Near-term Research Priorities*
    - Continue characterization of the transcriptional regulation of toxin genes
    - Genomic and proteomic analyses to describe the cofactors required for toxin expression, including possible promoters of toxin synthesis

    - Describe the dependence of toxin production on factors such as the availability of cofactors in the producing cells, as well as the stability and post-translational modification of biosynthetic enzymes
    - Characterize cyanobacterial phosphopantetheinyltransferases involved in toxin production
  - *Long-term Research Priority*
    - Determine effects of trophic interactions between bacteria, bacteriophage viruses, and algae on toxin production

- **Physiology**
  - *Long-term Research Priorities*
    - Describe the mechanisms of interactions with light availability, nutrient availability, grazers, and competitors by examining the expression of biosynthetic genes and cofactors in the target organisms
    - Integration of these studies with knowledge of the entire genome sequence would provide additional predictive power.

- **Toxin transport/fate**
  - *Near-term Research Priorities*
    - Describe toxin adsorption in environmental and water treatment martrices
    - Use overexpression, purification, and reconstitution of cyanotoxin-associated transporters such as McyH into membrane vesicles to enable us to demonstrate transport *in vitro* with radiolabeled toxin
    - Intracellular localization of McyH and other cyanotoxin-associated ABC transporters by *in situ* methods
  - *Long-term Research Priorities*
    - Development of techniques for observing the intracellular trafficking of all cyanotoxins, including development of specific toxin and transporter antibodies, as well as cyanobacterial imaging methods, are required

- **Role of toxins in ecology/physiology of toxic cyanobacteria**
  - *Long-term Research Priorities*
    - Characterize the roles of cyanobacterial toxins within the producing organisms by describing the molecular function of the specific toxin on the physiology and fitness of the producing microorganism, and on its influence on population structure and ecology of the given species

- Use functional genomic investigations to describe associations of toxin biosynthetic genes with cellular and metabolic responses to specific stressors
- Describe the roles of toxins in activities such as cyanobacterial quorum sensing and perception using protein expression studies to provide crucial information regarding the activation of metabolic pathways (e.g., phosphorylation of signaling proteins) associated with toxin production
- Confirm functional genomic data using mutagenesis and gene disruption techniques to elucidate the roles of toxins in the physiology of producing cyanobacteria
- Determine the role of protozoan predation in the natural selection of toxin-producing cyanobacterial species

Charge 7: Intentional/Accidental Release

Identify and prioritize research needed to reduce risks from an intentional or accidental release of cyanotoxins.

Several cyanotoxins have the potential to cause significant harm to human populations if intentionally or accidentally released through an environmental contamination event. The National Homeland Security Research Center (NHSRC) must now address critical issues that other programs may not have had to consider previously. Recent security interests have placed new and more pressing demands on the assessment of risks from exposure to biological agents through deliberate contamination. Distribution and possession of saxitoxin is already governed by Schedule 1 of the Chemical Weapons Convention as well as the Select Agent Rule. Therefore, saxitoxin and other cyanotoxins need to be evaluated for their risks to human health as well as their potential for intentional contamination. The high toxicity of several cyanotoxins at low concentrations warrants a thorough risk assessment of the most hazardous cyanotoxins. Research on exposure pathways and modes of delivery is needed to identify the cyanotoxins and the environmental conditions that present the greatest health risks.

Research Priority: Scientifically-sound risk assessments are needed to identify toxins with potential for use as terrorist agents

Data are needed to support scientifically-sound assessments of human-health risks from exposure to many cyanotoxins, particularly the risks posed by mixtures of cyanotoxins. Health risks should be based not only on exposure concentration, but also on route-of-exposure. Cyanotoxicity studies have focused primarily on toxicity resulting from oral ingestion. Much less is known about toxicity resulting from dermal and inhalational exposures. Many critical issues concerning mixtures and routes-of-exposure should be addressed, including bioavailability, interactions between toxins, direct modes of action, and indirect effects through immune system activation.

The risk assessment process involves parallel assessments of the hazard and exposure. However, additional parameters such as feasibility and vulnerability are required to assess risks from malevolent releases of biohazardous agents or materials. In the event of a release of a biological agent, the United States Environmental Protection Agency (USEPA) would respond in accordance with the Homeland Security Presidential Directives 5, 7, 9 and 10. The USEPA has mandated roles in decontamination, water infrastructure protection, and risk assessment. The risk associated with a deliberate exposure situation is one of the drivers for decisions regarding evacuation, decontamination, and eventual re-entry into a site or re-use of a water system. A contaminating event with cyanobacteria and/or cyanotoxins would require several layers of investigation. Contamination with saxitoxin (and potentially other cyanotoxins) would require distinguishing between a natural or intentional contaminating event. Risk-based decisions would need to be made concerning decontamination.

Several existing studies describing the chemical nature and characteristics of the saxitoxins are informative for risk assessments. Degradation and biotransformation of the saxitoxins have been investigated *in vitro* (e.g. Sullivan et al. 1983; Laycock et al. 1995), and in response to temperature (e.g. Lawrence et al. 1994), pH (e.g. Hall and Reichardt 1984; Gago-Martinez et al. 2001), combinations of temperature and pH (e.g. Ghazarossian et al. 1976, Indrasena and Gill 1999, Indrasena and Gill 2000). Saxitoxins in drinking water have been degraded using chlorine, and exposure to the treated solution did not result in increased cancer (Senogles-Derham et al. 2003). Removal of saxitoxins from drinking water using granular activated carbon, ozone, and hydrogen peroxide also has been examined (Orr et al. 2004).

The need to control access to potential terrorist agents such as saxitoxins is evident. However, the classification of toxins as potential terrorist agents causes restrictions to be placed on their distribution, thereby limiting research efforts. Efficient means for supplying sufficient quantities of cyanobacteria and cyanotoxins to researchers are needed because much of the data on which to base sound risk assessments are unavailable. Distribution efficiency can by increased by ensuring that requested quantities closely correspond to research (and commercial) needs. For example, laboratories developing reference standard materials may require larger quantities than research labs conducting physical or biochemical analyses. The regulations regarding handling should be clear and manageable to promote compliance. The most current information regarding registration and handling of select agents can be accessed via internet websites (http://www.opcw.org and http://www.selectagents.gov).

Early detection of cyanobacterial HABs and the presence of cyanotoxins in the environment, whether due to natural or intentional causes, is critical for public health protection. Improved techniques are needed to detect and identify HAB organisms, and to determine the potential for toxin production. Techniques also are needed to identify and quantify cyanotoxins, alone and in mixtures, and in various matrices. Molecular, genetic, and biochemical techniques hold promise for the development of techniques that are field-ready, rapid, reliable, and inexpensive. Homeland Security will benefit from methods that enable the presence of HABs and cyanotoxins in the environment to be classified as intentional or natural events.

Additional issues of importance to Homeland Security include the potential for bioaccumulation of cyanotoxins in plants, the aerosolization of cyanotoxins, and the induction of HABs in urban drinking water sources. Research has indicated the potential for cyanotoxin accumulation in agricultural crops when surface water contaminated with cyanobacteria is used for spray irrigation. Colonies and single cells of *Microcystis aeruginosa* and microcystin were retained by salad lettuce after spray irrigation with water containing microcystin-producing cyanobacteria (Codd et al. 1999). The uptake of toxins (pure as well as from cyanobacterial crude extracts) in different crop plants was also shown by Pflugmacher et al. (2006), Peuthert et al (2007 in press) and Järvenpää et al. (2007). Additional research is needed to characterize the risk posed by contaminated spray irrigation. Research is also needed to characterize the potential for aerosolization of cyanotoxins, the stability of cyanotoxins in various environmental matrices, the bioavailability of cyanotoxins bound to various matrices, the bioavailability of inhaled cyanotoxins, and cyanotoxin dermal transfer coefficients. Research is needed to assess the potential for in-

tentional induction of cyanobacterial HABs in surface waters, and to develop emergency risk management procedures.

Charge 7: Identify and prioritize research needed to reduce risks from an intentional or accidental release of cyanotoxins.

Near-term Research Priorities

- Scientifically-sound risk assessments are needed to identify toxins with potential for use as terrorist agents
  - Use appropriate dose-response data to define "significant quantities" below which there is no threat associated with the shipment and possession of cyanotoxins for research purposes
- Detection methods
  - Advances in monitoring for cyanobacteria and cyanotoxins are needed for early detection to prevent exposure
  - Biochemical and molecular techniques that yield detection signatures of cyanotoxins and cyanobacterial blooms are needed to discriminate natural versus unnatural contamination

Long-term Research Priorities

- Stability and degradation studies
  - Characterize cyanotoxin stability in various matrices, including food crops after contaminated spray irrigation
  - Characterize the physical and chemical parameters that enhance bioaffinity of toxin to various substrates
  - Characterize bioaccumulation and other natural concentration mechanisms
- Exposures other than oral
  - Describe changes in cyanotoxin modes of action based on exposure pathway and the presence of other cyanotoxins
  - Describe cyanotoxin aerosolization potential, stability, and bioavailability
  - Characterize the health risk of cyanotoxin inhalation, alone and in mixtures
  - Determine dermal transfer coefficients for cyanotoxins

Summary of Near-Term Research Needs for Highest Priority Cyanotoxins

Table 3 presents a summary of priority near-term research needs for the most prevalent cyanobacterial toxins. "Needs" boxes marked with a check (✓) indicate that little or no data is currently available for a given area of need. "Exist" boxes left blank indicate that the Workgroup was not aware of existing data or research efforts that address the specified area.

Table 3. Summary of Near-Term Research Needs for Highest Priority Cyanotoxins

Higher Research Priorities for the Given Charges	Current	MCY	CYN	STX	ATX
Protocols for efficient production, certification, and distribution of pure toxins and standards of consistent high quality	Need	Congeners	Deoxy	Lyngbya-related	✓
	Exist	LR	AWQC, NRC-IMB	FDA, NRC-IMB	
Study of toxicity from mixture components	Need	✓	✓	✓	✓
	Exist				
Labeled toxins	Need	✓	✓	Have	✓
	Exist	Laboratory Protocols		FDA,NOAA and IICH	
Toxin antibodies	Need	More	More	More	✓
	Exist	Commercial kits and antibodies	ELISA kits	ELISA kits	
Toxicokinetics -Whole animal (exposure route, distribution, metabolism, excretion) -Human in vitro (tissue, cell, enzymes) -PBPK modeling	Need	More	More	More	More
	Exist	Mice	Some	Rats & Cats	Some
Toxicodynamics	Need	More	More	More	More

Higher Research Priorities for the Given Charges	Current	MCY	CYN	STX	ATX
-In vitro screening for dose response effects to low, repeated, continuous exposures -In vivo screening for dose response effects to low, repeated, continuous exposures -Mixtures	Exist	Some	Some	Some	Some
Susceptibility Relies on knowledge from toxicokinetics and toxicodynamics	Need	More	✓	More	✓
	Exist	Age, gender		Some	
-Genomes or toxin gene clusters -Molecular marker for potential toxicity	Need	Have	Cylindrospermopsis	Lyngbya	Anabaena
	Exist	In progress (M.Anabaena)			
-Genetic regulation of toxin production and fate -Utilize for predictive models	Need	More	✓	✓	✓
	Exist	Some			
-Sound risk assessment to determine IF any of these toxins should be considered an intentional threat -Define significant quantities	Need	✓	✓	✓	✓
	Exist	Lots of data available	Limited data	Limited data	Limited Data

AWQC = Australian Water Quality Centre; FDA = US Food and Drug Administration; NRC-IMB = National Research Council Canada, Institute for Marine Biosciences; NOAA = National Oceanographic and Atmospheric Administration; IICH = International Isotope Clearing House

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