Chapter 25: Analytical Methods Workgroup Poster Abstracts

Early warning of actual and potential cyanotoxin production

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Introduction

The cyanobacteria which develop into mass populations in aquatic environments commonly include species and strains which produce potent toxins, alongside phylogenetically– or phenotypically similar strains which do not. The diverse range of low molecular weight cyanotoxins which can be produced present health hazards ranging from severe to mild in potable and recreational water resources.

Hypothesis

Risk management of these problems is aided if it is known whether the organisms present have the potential to produce cyanotoxins and whether they actually do so.

Methods

Understanding of the production and abundance of the toxins themselves is being advanced through the use of physicochemical and antibody methods. PCR is finding increasing application for the detection and quantification of cyanotoxin genes, whilst fluorescent *in situ* hybridisation (FISH) is

amenable for the localisation of cyanotoxin genes in mixed phytoplankton populations.

Results

Using such methods as an early warning system, we are quantifying microcystins in single filaments and single colonies of cyanobacteria using antibody–based procedures (CQ–ELISA) and measuring the genetic potential for microcystin and cylindrospermopsin production in single filaments and colonies through the use of PCR and FISH. Detection of DNA sequences for cyanotoxin peptide synthetases and polyketide synthases is thereby feasible in *Microcystis*, *Planktothrix*, *Anabaena*, *Nostoc*, *Nodularia*, *Aphanizomenon* and *Cylindrospermopsis*. Some examples are given for the United Kingdom.

Discussion

For these methods to be integrated into effective early warning systems, it is necessary that good systems are also developed for the collection and delivery of samples for analysis and for the rapid reporting of results. For early warning of potential or actual cyanotoxin production to be useful in cyanotoxin risk management, it is also necessary that data interpretation is available and that contingency measures by water utility managers and operatives are already in place.

Conclusion

Early warning of actual or potential cyanotoxin production is possible using a range of methods. Rapid sample processing times in a prepared laboratory can enable results to be obtained within as little as 1–3 hours, and typically on the day of sample receipt.

Detecting toxic cyanobacterial strains in the Great Lakes, USA

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Introduction

Some regions in the Great Lakes have been experiencing a resurgence of the cyanobacterial harmful algal bloom (HAB) genera *Microcystis*. Blooms of *Microcystis spp.* that produce the toxin microcystin have detrimental impacts on multiple levels, from disruption of zooplankton grazing to illness and mortality in animals and humans. Thus, it is of great concern that microcystin concentrations above the World Health Organization's recommended limit for drinking water (1 µg/L) have been measured in parts of Lake Huron and western Lake Erie, with particularly high concentrations in wind–accumulated scums. However, not all *Microcystis* strains produce toxins and traditional microscopic analyses are insufficient for discerning whether a bloom is composed of toxic strains. Instead, genetic analyses based on the *mcyB* gene, which is involved in cellular microcystin production, were used to differentiate toxic vs. non–toxic strains and specifically detect the presence of toxic strains of *Microcystis* in environmental samples.

Hypothesis

The use of *mcyB* gene can differentiate toxic and non–toxic *Microcystis* spp. that can potentially produce microcystin toxins.

Methods

Genetic analyses based on the *mcyB* gene, which is involved in cellular microcystin production, were used to differentiate toxic vs. non–toxic strains and specifically detect the presence of toxic strains of *Microcystis* in environmental samples.

Results

DNA sequence analysis of the *mcyB* gene revealed a genetically variable population of *Microcystis* in Saginaw Bay (Lake Huron) and western Lake Erie, with areas containing a greater proportion of toxic *Microcystis* strains also having higher microcystin concentrations, suggesting that changes in bloom toxicity may be the result of shifts in community composition. Another cyanobacterial HAB species, *Cylindrospermopsis raciborskii,* has also recently been detected in the Great Lakes and studies of its distribution and toxicity in this system are on–going.

Conclusion

The application of these methods to monitoring and modeling efforts will be important to protect human and ecosystem health in the Great Lakes region.

A progressive comparison of cyanobacterial populations with raw and finished water microcystin levels in Falls Lake Reservoir

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Introduction

Cyanobacteria and algal toxins have been placed on USEPA's drinking water Contaminant Candidate List (CCL2). Microcystin (MCYST), being the most frequently detected cyanobacterial toxin in water, is of high importance for study in potable water supply reservoirs. Processed water samples from numerous surface water supplies in the USA have been found positive for MCYST. MCYST has been detected in raw and finished water from Falls Lake, Raleigh, NC. Conventional drinking water treatment processes are only partially effective in removing cyanotoxins. To assess the risk of cyanotoxins in surface water supplies, USEPA is evaluating the use of cyanobacterial genera identification and enumeration. This study evaluated a known testing protocol and investigated the occurrence of MCYST in raw and finished water from Falls Lake Reservoir, as well as the relationship of toxin concentration to cyanobacterial populations.

Hypotheses

1. Applying standard sample extraction and concentration procedures increases the sensitivity of the Competitive–Binding ELISA assay.
2. Source water MCYST concentrations are directly related to cyanobacterial cell densities.

3. Water treatment processes used to treat Falls Lake raw water (pre–oxidation > coagulation/flocculation/PAC > sedimentation > filtration > chloramination) effectively remove MCYST from raw water.

Methods

From May 28–July 8, 2003, we collected raw and finished water samples from Falls Lake and Johnson WTP to assess the levels of microcystins. We applied standard sample preparation procedures, including: freeze–thaw/sonication; concentration via lyophilization; methanol–water extraction; and solid phase extraction. We then analyzed the concentrated samples using a commercially–available Competitive–Binding ELISA assay kit. Other aliquots of the water samples were preserved and analyzed by standard taxonomic and direct cell counting techniques.

Results

1. MCYSTs were detected above the assay limit of quantitation (LOQ)(0.160 ppb) in raw water concentrates.
2. MCYSTs in finished water concentrates were significantly lower, at or below the LOQ.
3. MCYSTs and the grouped densities of *Anabaena* and *Aphanizomenon* were weakly correlated ($R^2 = 0.11$).

Conclusions

1. For raw water samples, the sensitivity of the ELISA can be increased by using either an alternate low level protocol (LOQ 0.05 ppb) or pre–concentration techniques.
2. The low level protocol should be employed for finished water samples.
3. Conventional treatment processes removed 60–100% of MCYST.
4. *Anabaena* and *Aphanizomenon* densities may be useful predictors of MCYST levels.

Liquid chromatography using ion–trap mass spectrometry with wideband activation for the determination of microcystins in water

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Introduction

Microcystins are a chemically diverse group of heptapeptide toxins that are produced by cyanobacteria (blue–green algae) and over 65 have been characterised, to–date. Microcystins are specific inhibitors of protein phosphatases, are potent tumour promoters, and strict requlatory control is required to comply with the World Health Organisation guideline limit of 1 μg/L. Electrospray ion–trap mass spectrometry (MS) was applied to the determination of microcystins in cyanobacteria and water samples.

Hypothesis

Electrospray ion–trap mass spectrometry (MS) can detect and differentiate various congeners and analogues of microcystins in cyanobacteria and water samples.

Methods

Sample preparation involved C–18 solid phase extraction but large variations in extraction efficiencies were observed for individual microcystins, MC–LR, MC–YR, MC–RR and MC–LA. Both C–18 and amide columns were used for the separation of microcystins using liquid chromatography (LC) and both collision induced dissociation (CID) and MS/MS studies can been carried out simultaneously, using electrospray interfacing.

Results

Microcystins have a unique C–20 β–amino acid side chain (adda) and the cleavage of part of this moiety gives rise to a characteristic fragment ion at *m/z* 135 that allows the MS detection of unknown microcystins using source CID. MS studies revealed that the loss of a water molecule is typical of microcystins but to obtain abundant characteristic fragment ions from microcystins, WideBand activation together with a high collision energy was used. In this mode, both the parent ion, $[M+H]^+$ and the $[M+H-H_2O]^+$ ions were trapped and fragmentation of the latter produced spectral data that were characteristic of individual microcystins.

Conclusion

Liquid chromatography using ion–trap mass spectrometry with wideband activation can characterize individual microcystins in water.

Anatoxin–a elicits an increase in peroxidase and glutathione S–transferase activity in aquatic plants

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Introduction

Although the toxic effects of cyanotoxins on animals have been examined extensively, little research has focused on their effects on macrophytes and macroalgae. To date, only microcystins have been found to be detrimental to aquatic plants.

Hypothesis

Anatoxin–a elicits an increase in peroxidase and glutathione S–transferase activity in aquatic plants.

Methods

The peroxidase activity of the free floating aquatic plant *Lemna minor* and the filamentous macroalga *Chladophora fracta* was measured after exposure to several concentrations of the cyanotoxin, anatoxin–a. The effects of various concentrations of anatoxin–a on the detoxication enzyme, glutathione S–transferase (GST) in *L. minor* were also investigated.

Results

Peroxidase activity (POD) was significantly ($P < 0.05$) increased after 4 days of exposure to an anatoxin–a concentration of 25 μgmL^{-1} for both *L. minor* and *C. fracta*. Peroxidase activity was not significantly increased at

test concentrations of 15 μgmL^{-1} or lower. In another experiment, the effects of various concentrations of anatoxin–a on the detoxication enzyme, glutathione S–transferase (GST) in *L. minor* were investigated. GST activity was significantly elevated at anatoxin–a concentrations of 5 and 20 μgmL^{-1}. Photosynthetic oxygen production by *L. minor* was also found to be reduced at these concentrations.

Conclusion

This is the first report to our knowledge of the cyanotoxin anatoxin–a being harmful to aquatic plants.

The mis–identification of anatoxin–a using mass spectrometry in the forensic investigation of acute neurotoxic poisoning

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Introduction

Anatoxin–a (AN) is a potent neurotoxin, produced by a number of cyanobacterial species. Forensic investigations of suspected AN poisonings are frequently hampered by difficulties in detecting this toxin in biological matrices due to its rapid decay. Further impediments are the lack of availability of AN analogues and their degradation products. Possible confusion can also occur in identifying AN as the causative agent in both human and animal fatalities due to the presence of the amino acid Phenylalanine (Phe).

Hypothesis

Nano–electrospray hybrid quadrupole time–of–flight (nano ESI QqTOF) MS can accurately differentiate AN and Phe.

Methods

In July 2002 a suspected human intoxication in the USA, that relied on liquid chromatography–single quadrupole MS (LC–MS), confused Phe and AN, since both have similar masses. We previously developed a quadrupole ion–trap (QIT) MS for the determination of AN in cyanobacteria and drinking water. Liquid chromatography–multiple tandem mass spectrometry (LC–MS^n) was employed to study the fragmentation pathway of Phe, in positive mode, to identify characteristic product ions and fragmentation processes.

Results

Reversed–phase LC, using a C_{18} Luna column gave similar retention times and on certain C_{18} columns can co–elute. The molecular related species $[M+H]^+$ *m/z* 166 was used as the precursor ion for LC–MS^n experiments. MS^2–MS^4 spectra displayed major characteristic product ions for Phe. Fragmentation of other adduct ions $[M+Na]^+$ and $[M+NH_3]$ were examined in order to identify distinctive product ions. A comparison of the QIT MS^n data for AN and Phe can prevent misidentification. Nano–electrospray hybrid quadrupole time–of–flight (nano ESI QqTOF) MS was then used to confirm formulae assignments of the product ions using high mass accuracy data and to identify ions in the lower mass range.

Conclusion

Nano–electrospray hybrid quadrupole time–of–flight (nano ESI QqTOF) MS can confirm the formulae assignments of the product ions using high mass accuracy data and to identify ions in the lower mass range.

Cyanobacterial toxins and the AOAC marine and freshwater toxins task force

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Introduction

Cyanobacterial toxins have a significant economic and human health impact. Although there is a strong and global need for improved testing methods for these toxins, the demand for new, officially validated methods has not been met. Similarly, marine toxins require extensive monitoring programs and yet officially validated methodology is scarce or often based on outdated mouse bioassays. The AOAC Task Force on Marine and Freshwater toxins addresses this need by focusing efforts, setting priorities, and identifying economic and intellectual resources. The Task Force is an international group of experts on marine and freshwater toxins, and stakeholders who have a strong and practical interest in the development and validation of methods for detection of these toxins. The group establishes methods priorities, determines fitness for purpose, identifies and reviews available methodology, recommends methodology for validation, and identifies complementary analytical tools. Once appropriate analytical methodology has been identified or developed, the Task Force identifies financial and technical resources necessary to validate the methodology.

Since its first meeting in May 2004, the group has grown to include members from Europe, Asia, Africa, North and South America, and also members from Australia and New Zealand. As of September 2005, the group now totals 150 scientists, officials, and others. The toxins Task Force also has several members from the US state health agencies and federal agencies such as the Department of Health and Human Services (Food and Drug Administration, CFSAN Office of Seafoods, CFSAN/OC Shellfish Program, CVM), Department of Defense, Department of Commerce (National Oceanic and Atmospheric Administration), and Environmental Protection Agency.

New official method of analysis

The first product of the new AOAC Task Force is a new Official Method of Analysis for paralytic shellfish poisoning toxins (OMA 2005.6) which for the first time in over 45 years of shellfish monitoring, (saxitoxins) has been approved allowing an alternative to animal testing that will have a worldwide impact. The new method, developed by Health Canada and based on precolumn– oxidation HPLC and fluorescence detection, has also found some application to saxitoxins–producing cyanobacteria.

Cyanobacterial toxins

Although the first year emphasized the marine toxins, efforts addressing cyanobacterial toxins are rapidly increasing with the appointment of many experts and stakeholders in this field. Many oral and poster presentations addressing the cyanobacteria and associated toxins were presented, along with marine toxin presentations, at the group's first major conference, "Marine and Freshwater Toxins Analysis: 1st Joint Symposium and AOAC Task Force Meeting" in Baiona, Spain. At this unique meeting, participants discussed test kits for microcystins and validation needs for the cyanobacterial toxins, in general. The high level of interest recently led to the formation of a new cyanobacterial toxin subgroup.

New subgroup to address cyanobacterial toxins

The new cyanobacterial subgroup, chaired by Task Force member Hans van Egmond (co–chairs to be appointed) will meet for the first time at AOAC's Annual meeting in Orlando, Florida on Sept. 2005, as will several marine toxin subgroups. Also, included in the extensive toxins program for Orlando are two symposia addressing marine and freshwater toxins including presentations on topics in cyanobacterial toxin detection. The Task Force intends to interact extensively with the US EPA and Dr. Armah de la Cruz will also present in Orlando a brief overview of methods discussions from ISOC–HAB. Also planned are Task Force relevant presentations on cyanobacterial toxins and marine toxins at symposia to be held at Pacifichem 2005 in Honolulu, Hawaii, Dec. 2005.

Detection of Toxic Cyanobacteria Using the PDS® Biosensor

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Introduction

Cyanobacteria can produce molecules hazardous to human health (i.e. hepatotoxins and neurotoxins). The ubiquity of cyanobacteria in terrestrial, as well as freshwater and marine environments, suggest a potential for widespread human exposure. We have developed a Pathogen Detection System (PDS®) biosensor to monitor the presence of toxic cyanobacteria in freshwater. This biosensor allows the detection of live bacteria in water and biological fluids, as well as, the detection of cytotoxic compounds on mammalian cells.

Hypotheses

Biophage Pharma Inc., in collaboration with the NRC/BRI, has developed a patented PDS® biosensor based on impedance (for more details visit www.biophagepharma.com). The PDS® biosensor allows the detection and quantification of living pathogens in water and biological fluids. It can also assess cytotoxicity on mammalian cells (normal and cancer cells). As toxic cyanobacteria emerge as potentially hazardous microorganisms for human, animal and marine health, the PDS biosensor can be used to monitor the presence of cyanobacterial cells in drinking waters. In addition, cyanobacterial toxins should be monitored in cyanobacteria–positive samples as well as in invertebrates, fish or grazing animals used for human consumption.

Methods

Samples are mixed directly with broth media, added in the PDS® wells and then monitored for up to 24 hrs.

Results

The PDS® biosensor can detect and quantify with great precision a small number of bacteria (about 5 bacteria/ml) without any pre–amplification step. With the addition of a small pre–amplification step, this limit could be lowered without modifying the total time from sample collection to detection. At very low concentrations, the total time for detection varies between 2 h for fast growing bacteria, to 24 h in very slow growing bacteria, which is at least two times faster than conventional culture techniques. In addition, detection is monitored in real time on a computer screen allowing for immediate action as soon as detection occurs.

Conclusions

The PDS® biosensor allows the detection of a large number of organisms two times faster than conventional culture techniques. The samples do not require preprocessing and can detect very low number of bacterial cells (5 cells/ml). In addition, detection can be monitored in real time on a computer screen allowing for immediate action as soon as detection occurs. The PDS® biosensor can also detect cytotoxicity on mammalian cells.

Development of microarrays for rapid detection of toxigenic cyanobacteria taxa in water supply reservoirs

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Introduction

Reservoirs are the principal supplies of drinking water for many urban areas and also serve as major recreational areas. In North Carolina, reservoirs are generally characterized by high nutrient levels, high turbidity, high total organic carbon, and low alkalinity. They also commonly develop cyanobacterial blooms which can comprise more than 90% of the phytoplankton cell number at times. Dominant cyanobacteria taxa include *Cylindrospermopsis* spp. (including *Cylindrospermopsis raciborskii*), *Anabaenopsis, Planktolyngbya limnetica, Aphanocapsa* sp., *Aphanizomenon gracile, Oscillatoria, Anabaena, Microcystis,* and *Aphanizomenon*, and microcystins have been found in raw water from these reservoirs.

Materials and Methods

We are developing microarray detection methods for assessment of cyanobacterial taxa in these freshwater systems. In previous work, our prototype arrays were able to detect the DNA of heterotrophic bacteria, eukaryotic protists and cyanobacteria in samples of genomic DNA extracted from three different lakewater samples with a high degree of sensitivity and specificity. To expand the prototype array for cyanobacteria our first step has been to identify cyanobacterial taxa of interest through the literature and field data. Second, cyanobacterial gene libraries were constructed from four representative NC reservoirs using PCR with primers that target cyanobacterial small subunit ribosomal DNA (SSU rDNA) genes. Third, taxon–specific (generally species or clone–specific) PCR primers and 50–mer oligonucleotide probes are designed to complement unique sequences in the variable regions of the SSU rDNA. Fourth, the oligonucleotide

probes are printed onto glass slides to form the microarray. Fifth, using both the microarray and real–time PCR, probes are tested for specificity and sensitivity. Finally, the microarray will be used to assess the presence and relative abundance of targeted cyanobacterial taxa over a three year period in NC reservoirs. Microarray results will be compared to direct microscopic count assessments. An additional aspect is that we are also developing oligonucleotide probes for the array that target known toxin biosynthesis genes (e.g. microsytin and cylindrospermopsin synthesis genes).

Conclusion

At present, we are testing our first group of primers and oligonucleotide probes targeting 25 known cyanobacterial taxa (at the species or sub–species level) and 17 novel cyanobacterial clones derived from the NC reservoirs prior to spotting on arrays and field testing. We anticipate that microarrays will be a powerful tool with the potential to assess the abundance of cyanobacterial in near real–time. Such data will aid in management decisions to prevent or mitigate the effects of cyanobacterial blooms.

ARS research on harmful algal blooms in SE USA aquaculture impoundments

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Introduction

In the United States, catfish aquaculture accounts for around 70% of the total freshwater revenue (currently around $1 billion annually). During the 15–18 month stocker–sized fry to fillet turnover, effusive algal growth results from the high stocking and feeding rates. Approximately 1.5 mg/L N in the form of unassimilated feed and fish excreta is added to ponds on a daily basis. Pond management must contend with maintaining algal blooms to process this nitrogen while preventing bloom collapse and hypoxic to anoxic dissolved oxygen conditions. In 1999, an Agricultural Research Service (ARS) research unit was formed to assess optimal pond management scenarios for fish production. This program can be divided into two major components: 1) prediction of harmful algal bloom events with the goal of identifying forcing variables leading to bloom events and 2) development of rapid assessment technologies for pond management.

Methods

Microcystin, anatoxin–a, euglenophycin, and prymnesin toxins have been previously reported from this research unit. Fish mortalities have been documented from these blooms in five southeastern states (AR, LA, MS, NC, SC, and TX). In 2000, a synoptic survey of 3% of the total production ponds was conducted in the southeastern 4–state catfish production area after documentation of microcystin–fish mortalities. Water samples were collected from 485 production ponds during a 10–day period, with analyses of pigments, off–flavor, and microcystin toxins (using HPLC/MS). A PCR method using myc b gene was developed to identify algae capable of microcystin synthesis.

Detection of algal blooms is difficult in freshwater, principally due to the alteration of reflectance from algae by suspended solids and color. At present, no modeling software is designed for use in Case II waters. Zimba and Gitelson (in review) have proposed tuning model properties to water column conditions to better estimate standing stock biomass (chl a) and applied this model to aquaculture ponds. The conceptual three–band model [R–1(l1)–R–1(l2)]×R(l3) and its special case, the band–ratio model R(l3)/R(l1), were spectrally tuned in accord with optical properties of the media and optimal spectral bands (l1,l2, and l3) for accurate chl *a* estimation were determined.

Results

Myxoxanthophyll, a cyanobacterial carotenoid biomarker, was strongly correlated with microcystin content in the synoptic survey (R= 0.92). Microcystin was detected in over 50% of all ponds sampled, with WHO limits exceeded in <1% of surveyed ponds. Myxoxanthophyll is present only in coccoid cyanobacteria and is a useful first approximation of potential toxic episodes. The PCR method had a sensitivity resolution of *ca*. 10 cells and was able to detect toxic algae at microcystin concentrations >0.25 ng/mL.

The same technique of model tuning (l1 for phycocyanin, l2 for chl) is being used for modeling cyanobacterial biomass, rather than indirect ratio methods currently used. Application of Case1 water chl model to freshwater pond data resulted in poor model fit (40–60% explained variance). This new method improved model accuracy by 14%.

Conclusions

Toxic cyanobacteria blooms occur in over 50% of aquaculture impoundments. Development of spectral models for Case 2 waters will serve as a management tool for assessment of cyanobacterial blooms.