Overview of U.S. EPA Office of Research and Development's planned research on analysis and monitoring in fresh and coastal/estuarine environments.

Heath Mash, James Lazorchak, Toby Sanan, Tammy Jones-Lepp, Jade Morgan, Michael Elovitz, Joel Allen, Jingrang Lu, Jorge Santodomingo, Robert Zucker. Armah de la Cruz, U.S. EPA Office of Research and Development

United States Environmental Protection Agency

Several factors are contributing to the development of the "perfect" Harmful algal Bloom (HAB) storm. For example, climate change associated with elevated temperatures over prolonged time periods, changes in population demographics, agricultural land use linked to nitrogen loading increases, chronic economic stress and an aging water treatment infrastructure all combine to increase the probability of toxins breaking through to consumers' taps.

Increases in salinity are also having adverse ecosystem impacts stemming from freshwater HABs and invasive toxic algae. The most problematic of these is the marine invasive Prymnesium parvum (i.e., "golden algae") which has caused fish kills in Texas annually since 2001 and has been documented in at least 10 other states.

This research plan has several objectives:

1) develop new or refine existing chemical, instrument and biological methods for the detection of cyanobacteria and their toxins; test such methods in field studies in both HAB and non HAB environments;

2) determine the method(s) that can be best uses as early warning (pre bloom conditions within days to weeks) systems for the detection of cyanobacteria and their toxins.

We will discuss ongoing efforts of 8 research projects that include chemical detection of toxins, use of molecular, flow cytometry, mass spectrometric, 2 phone apps 1) HABs early warning app for estimating the percentage of bluegreens vs other algae, 2) microscopic approaches for phyto/zooplankton identification, and advanced instrumental and hyperspectral image analysis approaches.

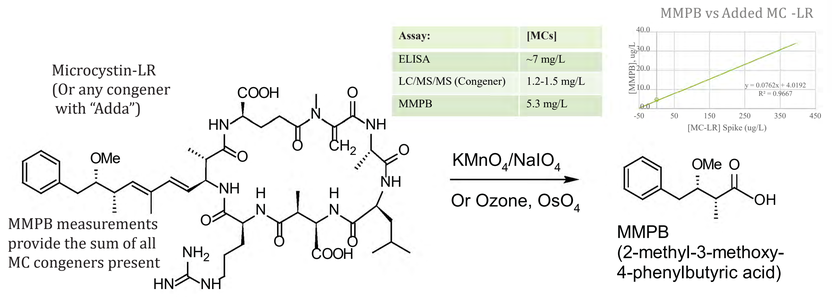
Research Effort 1

Development of analytical methods for improved measurement and monitoring of toxins related to harmful algal blooms (HABs)

Heath Mash, mash.heath@epa.gov; Toby Sanan, sanan.toby@epa.gov

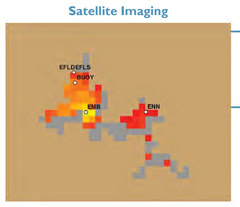
Research progress to date:

A technique for measuring "total" microcystins, rather than individual congeners, using LC/MS/MS techniques in conjunction with chemical derivatization (MMPB method) has been applied to multiple ongoing studies of HAB events within Ohio as well as in EPA Region 8. The results are being compared with ELISA, qPCR, conventional LC/MS/MS, and toxicity screenings. The results suggest that the diversity of MC congeners in a given bloom strongly influences the response by conventional LC/MS/MS methods, and that the MMPB method when applied to surface waters can bridge the gap and provide secondary confirmation of toxin measurements obtained using ELISA.



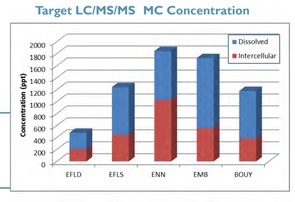
Assay:	[MCs]
ELISA	~7 mg/L
LC/MS/MS (Congener)	1.2-1.5 mg/L
MMPB	5.3 mg/L

2017 Lake Harsha Monitoring Study



Estimated Cell Counts
EFLD/EFLS = 1,023,293 cells/ml
BUOY = 676,083 cells/ml
EMB = 323,594 cells/ml
ENN = 1,258,925 cells/m

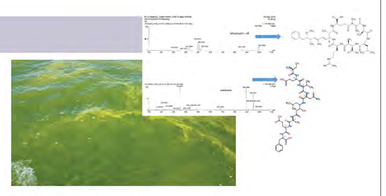
Total Target MC Concentration
EFLD/EFLS =1244.3 ppt (IC 446 ppt)
BUOY = 1179.7 ppt (IC 383 ppt)
EMB = 1725.3 ppt (IC 560 ppt)
ENN = 1843.8 ppt (IC 1032 ppt)



Research Effort 2

Developing new analytical tools to assist in the characterization of the formation of *P parvum*, and other algal toxins.

Tammy Jones-Lepp; Jade Morgan, organ.jade@epa.gov

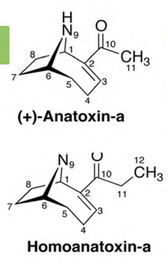


Lake Havasu cyanobacterial bloom pictures courtesy of Dr Doyle Wilson, City of Lake Havasu water manager

Research progress to date:

Methods for the potent algal toxins: anatoxin-a and prymnesins.

- Modified EPA drinking water methods 544 and 545 for use in source waters.
- Anatoxin-a was detected in Klamath river samples up to 7 ng/mL concentrations before degradation.
- Prymnesins were detected using a robust extraction technique and confirmation ions



(+)-Anatoxin-a

Homoanatoxin-a

Hehlkeek 'We-Roy picture courtesy of NPS website

Research Effort 3

Development of methods for assessing tissue levels of Algal Toxins in Aquatic Food Webs.

Jim Lazorchak, NERL/EERD lazorchak.jim@epa.gov; Toby Sanan sanan.toby@epa.gov

Research progress to date: See Platform presentation 237 Tuesday 10AM.

Sampling and laboratory work underway – 'total' microcystin extaction method (MMPB) has been developed and tested using 3 microcystin congeners. MMPB method successfully used to detect microcystins in spiked fish tissue samples and in fish exposed to microcystins in an experiment stream facility phosphate study.

- Spikes at high (40 ng) and low (4 ng) MMPB and MMPB -D_3 were performed to evaluate extraction performance.
- Consistent recovery with low and high fish samples, and for 4 and 14% lipid samples.
- Recovery of MMPB in 'blank' samples (9, 10) shows stability under derivatization conditions even in low background matrix.

MMPB Application to Fish Tissue – MMPB Spike/Recovery Studies

Sample #	MMPB Spike (ng):	MMPB-D3 Spike (ng)	Fish (mg)	Lipid %	MMPB % Recovery
1	40	40	10	4	85
2	40	40	100	4	102
3	40	40	10	14	84
4	40	40	100	14	73
5	4	4	10	4	81
6	4	4	100	4	61
7	4	4	10	14	87
8	4	4	100	14	79
9	40	40	0	na	102
10	4	4	0	na	83

Research Effort 4

Toxic Algae *in-vivo* fluorescence sensor

Michael Elovitz, elovitz.michael@epa.gov; Joel Allen, allen.joel@epa.gov; Jim Lazorchak, lazorchak.jim@epa.gov

Research progress to date:

Studies are underway to explore the presence of accessory pigments within Prymnesium parvum that could be useful for selective in vivo identification and quantitative/semi-quantitative enumeration of P. parvum in an aquatic environment. Working with lab-grown mono-cultures of P. parvum we are examining the in vivo fluorescence excitation-emission matrix of suspended P. parvum. Preliminary studies have not shown readily identifiable fluorescence peaks that are both unique to P. parvum, and have a fluorescence intensity that would allow facile identification at typical environmental concentrations. On-going work will focus on the carotenoid region of the fluorescence matrix.

Research Effort 5

Toxic Species identification using eDNA, traditional identification methods, Toxic Algae Identification App and Toxic Gene-specific Monitoring for Harmful Algal Bloom using Meta-transcriptomic and RT-qPCR Approaches

Jingrang Lu, lu.jingrang@epa.gov ; Jim Lazorchak, lazorchak.jim@epa.gov

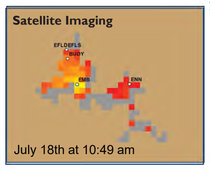
Research progress to date:

- **Assays:** A panel of SYBR-qPCR assays to detect different levels of toxic and non-toxic cyanobacteria was used for Lake Harsha samples, although we are still working on new development and improvement of the assays.
- **qPCR:** Indicated the biomass, variation and bloom development in Lake Harsha.
- **RT-qPCR:** Good indicators for toxic cyano and even toxin variations in Lake Harsha. They could be used to indicate the initiating/ending and peak period of toxin in raw water.

- *Microcystis aeruginosa* was the major species responsible for MC production, while *Anabaena circinalis* was responsible for anatoxin production, although the other toxic groups: *Aphanizomenon, Cylindrospermopsis, Nostoc, Limnothrix, Lyngbya, Oscillatoria* and *Planktothrix* were also determined.

Analysis of microbial metagenomic transcriptional responses during cyanobacterial bloom revealed that Nostocales (mainly Anabaena) dominated in the early stage of the cyanobacterial bloom closely associated with nitrification and nutrients in water, and then Microcystis dominated the community during bloom and caused the increase of toxin and decrease of nutrients, implying the key role for nitrogen and phosphorus.

- An HABs app has been under development and updated. One site on Harsha Lake has a camera setup and has been collecting pictures since September, 2016.
- In 2017, Two camera locations on the Ohio River have been setup in the Cincinnati Area.
- As time of day changes as well as seasons, some differences were found at both the lake site and river sites

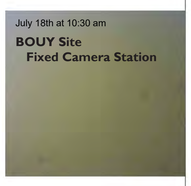


Courteous of Jim Lazorchak/Blake Schaeffer

2017 Lake Harsha Monitoring Study Comparing Methodologies

Estimated Cell Counts
EFLD/EFLS = 1,023,293 cells/ml
BUOY = 676,083 cells/ml
EMB = 323,594 cells/ml
ENN = 1,258,925 cells/ml

Fixed Camera Prediction of Cyanobacteria
- Camera Prediction:
- 10:30 am - 98.3% Probability of Bluegreens
- 11:30 am 100% Probability of Bluegreens



Research Effort 6

The use of a polyphasic approach to track the presence of cyanobacteria-producing toxins, toxin production, and toxin inactivation.

Jorge Santodomingo, santodomingo.jorge@epa.gov

Research progress to date:

Goal Purpose: To develop a molecular toolbox that can identify and detect cyanobacterial and noncyanobacterial targets linked to toxin production

Evaluation of commercially available qPCR kits used for the detection of total cyanobacteria and cyanobacterial toxin genes in Ohio surface waters (in collaboration with Ohio-EPA). Understanding the dynamics of eukaryotic and prokaryotic communities and assessing their potential value as predictors of cyanobacterial blooms.

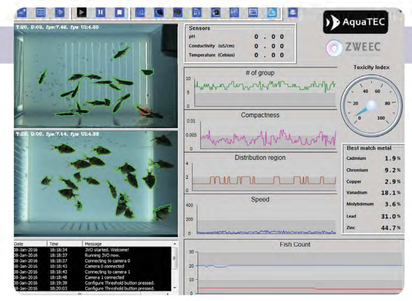
Ongoing studies: 16S and 18S rRNA and rRNA gene sequencing using next generation molecular methods to look at the composition and structure of microbial communities associated with cyanobacterial blooms

Results: Using sequencing analysis of the 16S rRNA gene, we have identified 11 different cyanobacterial genera in samples collected from Lake Harsha (2015). Among the most dominant genera are *Cylindrospermopsis, Dolichospermum*, *Microcystis*, and *Planktothrix.* Other abundant bacterial groups are Bacteroidetes, Chlorobi, and Chloroflexi. RNA libraries suggested that most of the active organisms in these samples were cyanobacteria, Increases in densities and activity of these groups were noted early summer. Additionally, potential cyanobacterial predators (i.e., copepods) and a large diversity of phytoplankton were identified in 18S rRNA sequencing libraries.

Research Effort 7

Development and Standardization of Real time Online Toxicity Monitors (OTM).

Joel Allen, allen.joel@epa.gov; Jim Lazorchak, lazorchak.jim@epa.gov

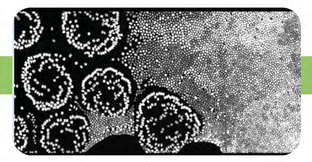


- **Cooperative Research and Development Agreement between EPA/ORD and ZWEEC Analytics finalized**
- **Goals**
  - –Evaluation of a fish OTM, AquaTEC, through benchmark testing
  - –Investigation of alternative fish species
  - –Investigation of the use of AquaTEC in a source water monitoring context
- **Approach**
  - –Perform laboratory challenges designed to provide information on sensitivity of AquaTEC
  - –Assay multiple species including Tiger Barb (Puntigrus tetrazona), Fathead Minnow (Pimephales promelas), and Zebra Fish (Danio rerio)
  - –Field deployment of the AquaTEC system will investigate its potential use in a source water context with particular interest in detection of Harmful Algal Blooms
  - –Data produced will inform the most appropriate use and extent of the capabilities of the AquaTEC system while providing valuable feedback for the improvement of event detection algorithms

Research Effort 8

Algae at the Crossroads: Detecting environmental changes in water using flow cytometry, microscopy and hyperspectral image analysis.

Robert Zucker zucker.robert@epa.gov

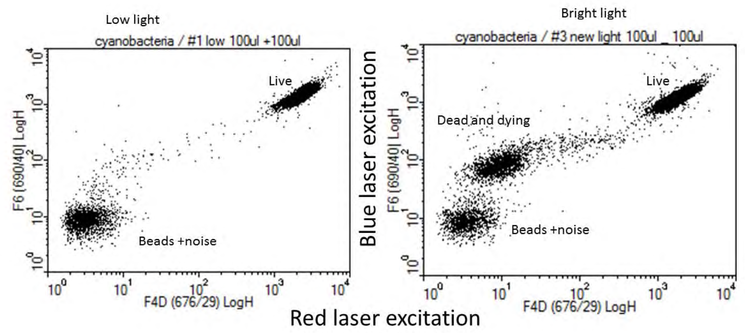


Research Progress to date:

Photosynthetic picoplankton is the fraction of the phytoplankton performing photosynthesis composed by cells between 0.2 and 2 µm (picoplankton). As lake water loses nutrients, an abundance of small freshwater picoplankton appear. Using the flow cytometer we can quantify picoplankton relative to a known concentration of 1-2um fluorescent beads.

PARISS (Prism and Reflector Imaging Spectroscopy System), is used to measure the absorption/emission characteristics from pigments and chlorophyll contained within live algae and cyanobacteria. We have used hyperspectral imaging to create a spectral data base of algae and cyanobacteria. This spectral data was used in conjunction with standard microscopic morphology was useful for the identification and classification.

A fluorescent microscope method was developed to rapidly distinguish cyanobacteria from algae. The difference between algae and cyanobacteria is based on their autofluorescence of their unique photosynthetic pigments. By combining images obtained using two fluorescence excitation wavelengths, we can quickly visualize the different organisms. The morphological identification of organisms was then confirmed using the PARISS hyperspectral imaging system.



Flow cytometry was used to study the growth and death of microcystis. As microcystis die both their fluorescence and light scatter decreases. Picoplankton (1-2 micron) could be distinguished from microcystis by fluorescence and scatter parameters.

Research Effort 9

Determination of Microcystin Toxicity *in Vitro* in HepaRG Human Hepatocytes.

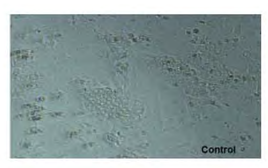
Armah de la Cruz; delacruz.armah@epa.gov

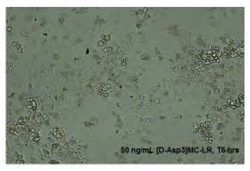
Research Progress to date:

Microcystins are a family of cyanobacterial toxins (cyanotoxins) common in freshwater worldwide during harmful bloom events. In mammals, the liver is the primary target organ but can also affect other organs and tissues. Analytical methods to detect these toxins in samples are limited and may not necessarily reflect overall toxicity. The determination of toxicity is essential in risk assessment and proper management of water resources. An in vitro cell culture cytotoxicity assay was developed using a differentiated human liver cell line, HepaRG. Cytotoxic effects are measured visually and biochemically using ATCC XTT Cell Proliferation assay. Pure individual cyanotoxin is initially analyzed for cytotoxicity. Results showed that hydrophobic microcystins are more cytotoxic than less hydrophobic variants (1->1000 ng/mL endpoints). Cytopathic effects can be observed microscopically as short as 30 min post exposure. In mixture studies with MC-LR and another congener, preliminary results showed toxicity below the endpoints. Environmental water samples were screened initially by ELISA for total microcystins. Preliminary results showed the cytopathic effects (CPE) and ELISA are weakly correlated (r2= 0.22) and the ELISA/CPE ratio ranges from 1-100.

Cytopathic Effects (CPE)

- Blebbing
- Refractile
- Amorphous
- Enlarge
- Rounding
- Clumping
- Detach (if cells attach)
- Granular
- Cell death





Next Steps

Research Effort 1
Development of analytical methods for improved measurement and monitoring of toxins related to harmful algal blooms (HABs)

Determine the variability of possible algal products using Non-Targeted analysis with High-Resolution Mass Spectroscopy

Research Effort 2
Developing new analytical tools to assist in the characterization of the Prymnesins

Since there is no available neat standard, further research is needed to validate the concentrations and stability of the samples. In full scan mode we see a few confirmatory masses, however further method development is needed to isolate the compound.

Research Effort 3
Development of methods for assessing tissue levels of Algal Toxins in Aquatic Food Webs

Fathead minnows exposed in our Experimental Stream Facility Nitrate/Phosphate dosing will be analyzed after dosing to determine if they bioaccumulate microcystins produced by periphytic benthic cyanobacteria

Research Effort 5
Toxic Species identification using eDNA, traditional identification methods, Toxic Algae Identification App and Toxic Gene-specific Monitoring for Harmful Algal Bloom using Meta-transcriptomic and RT-qPCR Approaches

Toxic Species identification using eDNA, traditional identification methods, Toxic Algae Identification App and Toxic Gene-specific Monitoring for Harmful Algal Bloom using Meta-transcriptomic and RT-qPCR Approaches

–New assays developed will be further tested, validated, published and used in different water samples
–Apps: HABs Early Warning - Summer of 2017 finalize camera stations on the Ohio River and at EPA's North Carolina campus. Collect samples and calibrate camera app.
–2018 Plankton ID app will start to be developed

Research Effort 6
The use of a polyphasic approach to track the presence of cyanobacteria-producing toxins, toxin production, and toxin inactivation

The use of a polyphasic approach to track the presence of cyanobacteria-producing toxins, toxin production, and toxin inactivation. Development of novel qPCR assays to monitor cyanobacteria, zooplankton and phytoplankton species in different lakes.

Research Effort 8
Algae at the Crossroads: Detecting environmental changes in water using flow cytometry, microscopy and hyperspectral image analysis

Quantify cell number and percentage of cell death from Microcystis Aeruginosa (cyanobacteria) using flow cytometry. Correlate state of culture to production of toxin.

Grow Microcystis Aeruginosa (cyanobacteria) under different light conditions (blue, orange and red LED lights) to correlate cell number and death with toxin production

Rapidly detect differences between algae/ cyanobacteria using microscopic fluorescence.

Study Picoplankton growth using flow cytometry and hyperspectral imaging and connect to possible production of toxin.

Investigate biophysics parameter changes during growth of Microcystis Aeruginosa (cyanobacteria) using hyperspectral imaging and flow cytometry.

Research Effort 9
Determination of Microcystin Toxicity in Vitro in HepaRG Human Hepatocytes

Studies are underway using mixtures of pure cyanotoxins. Results obtained in this study will be compared with existing conventional methods.