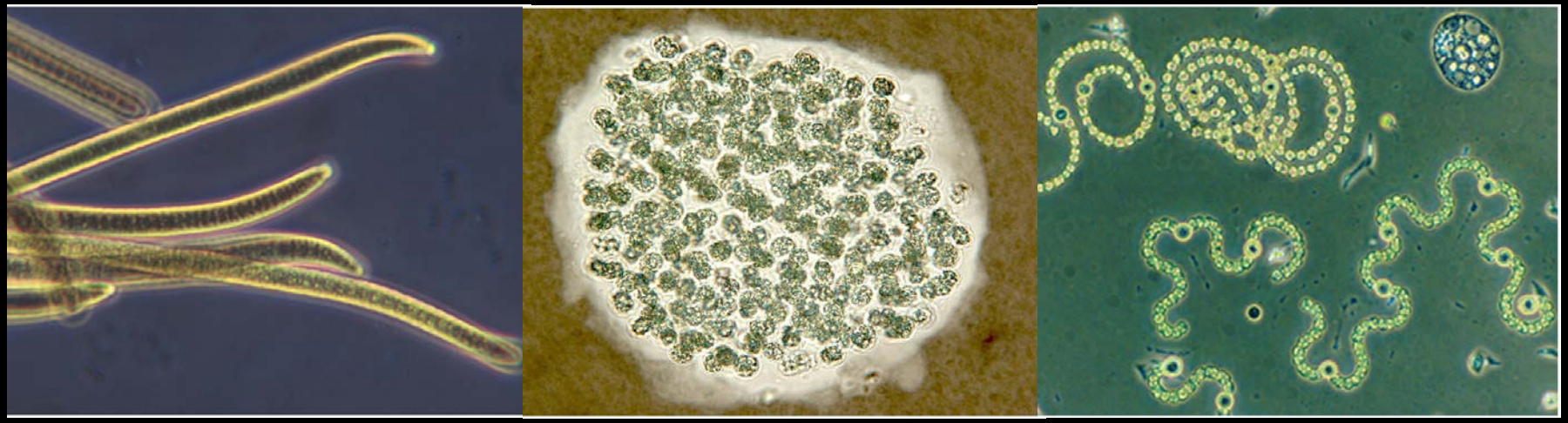
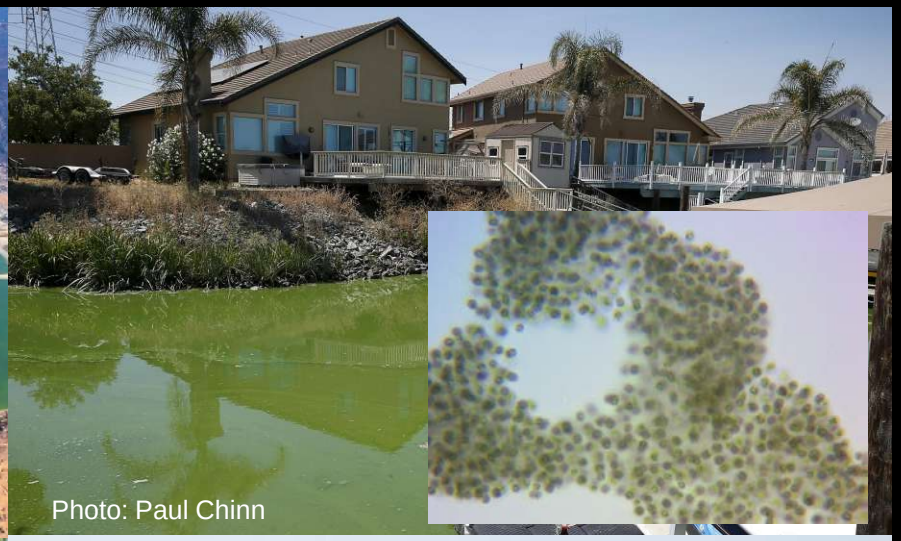
Presentation overview

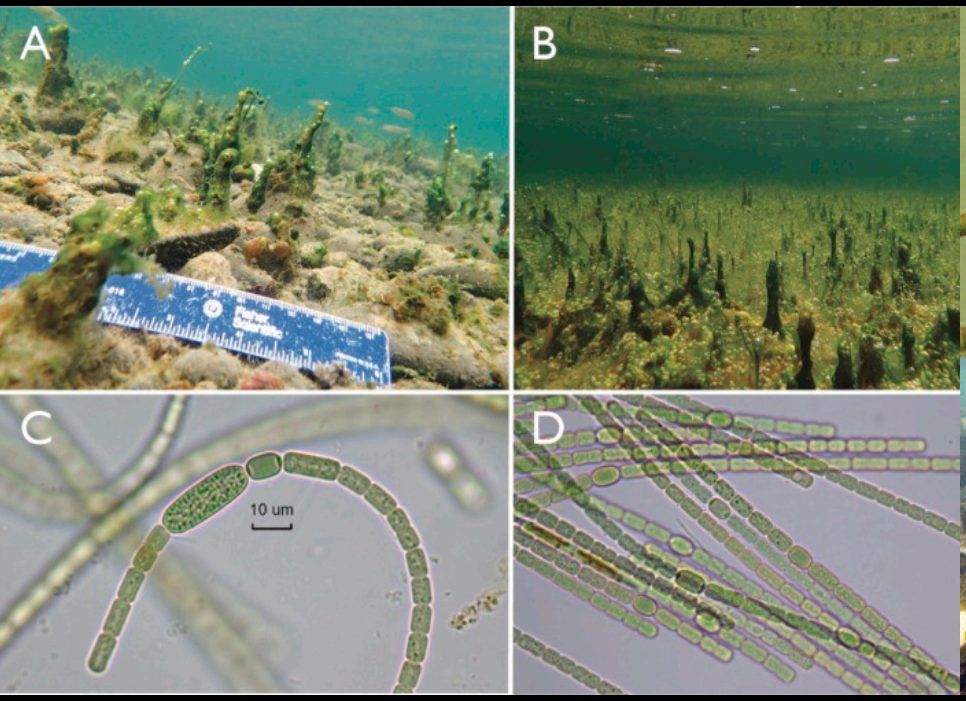
- Overview of planktonic and benthic cyanobacteria and cyanotoxin biosynthesis → a strain level trait!
- Sample collection/processing for DNA based analyses
- Principles of commonly used molecular techniques
  - Real-time quantitative polymerase chain reaction (QPCR)
  - DNA barcoding/amplicon sequencing
  - Shotgun metagenomics
- Examples of QPCR as part of a tiered monitoring framework
- Pros and cons of each approach



CyanoHABs are an increasingly common occurrence in many freshwater systems

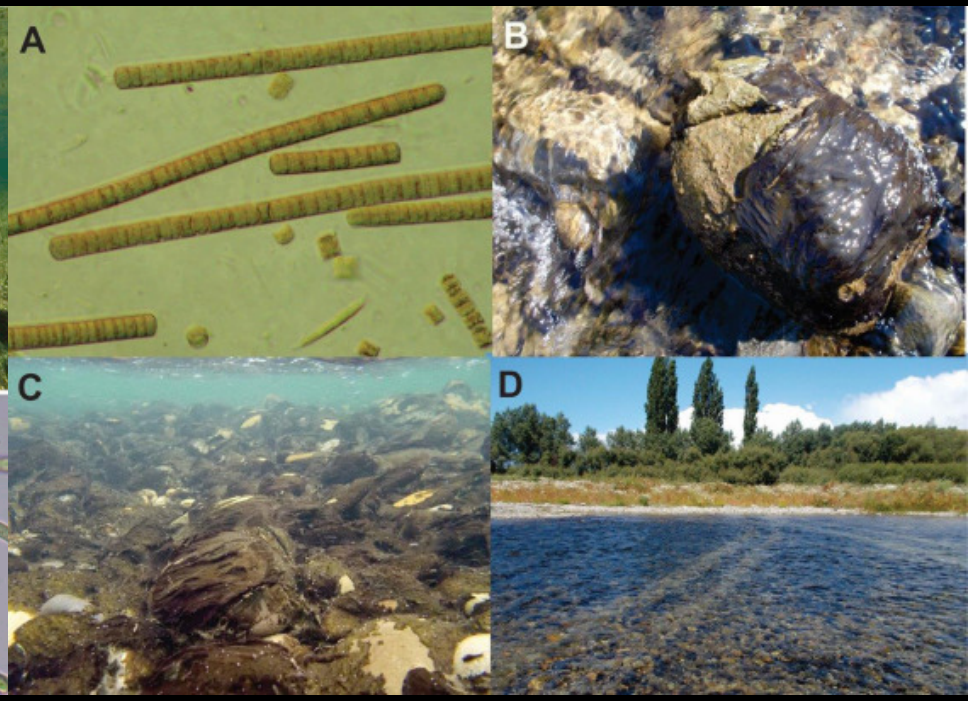


Benthic & periphytic CyanoHABs



Benthic *Anabaena* sp. – Eel River, CA

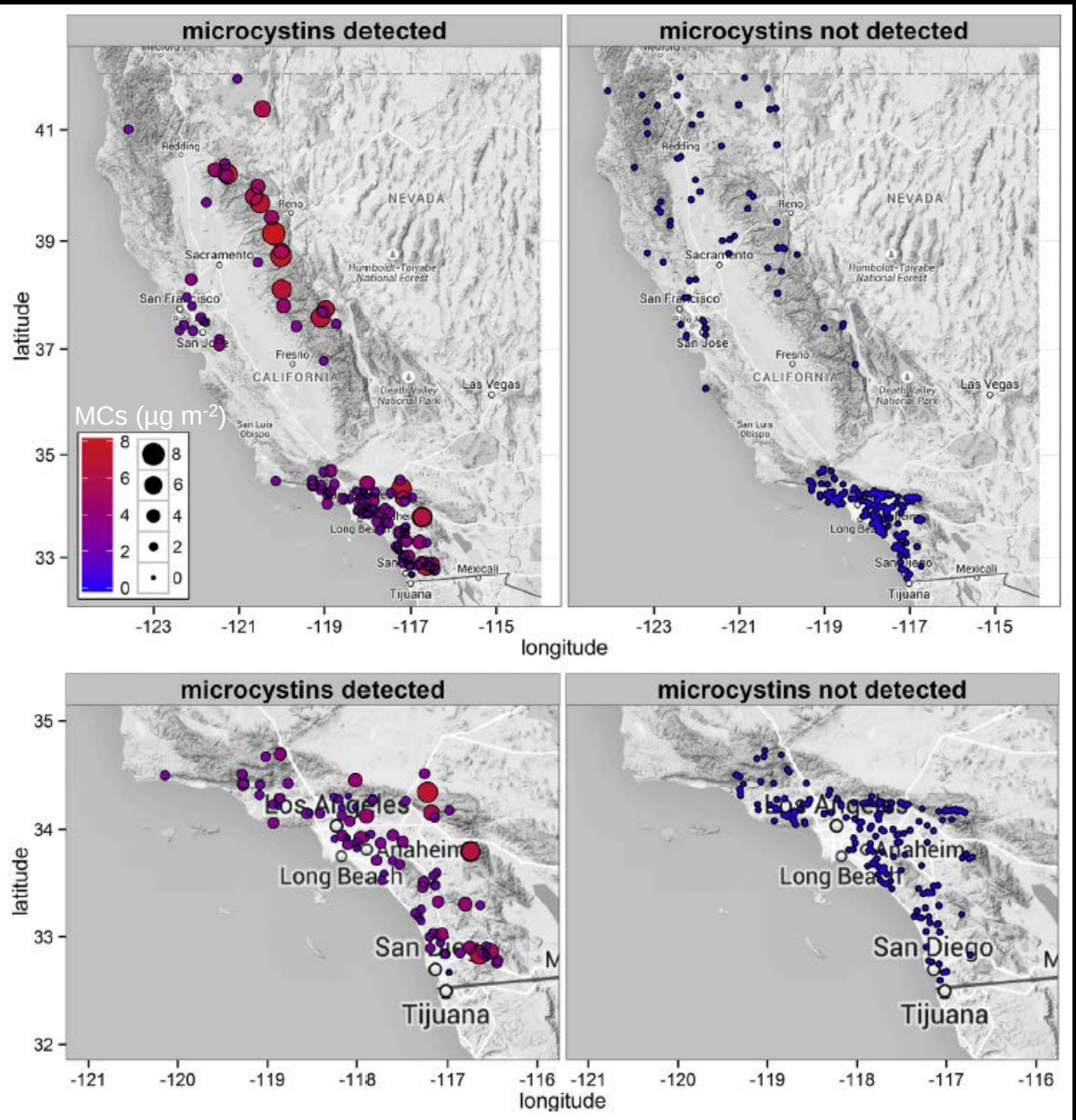
Bouma-Gregson et al., 2017.
Harmful Algae 66:79-87



Benthic *Phormidium* sp. – New Zealand

McAllister et al., 2016.
Harmful Algae 55:282-294.

Benthic cyanos in wadeable streams



Dominant taxa were *Nostoc*, *Leptolyngbya*, *Anabaena* and *Phormidium* (2011-2013 yrs).

33% tested positive for MCs, 21% for lyngbyatoxin, 7% STX, 3% ANTX

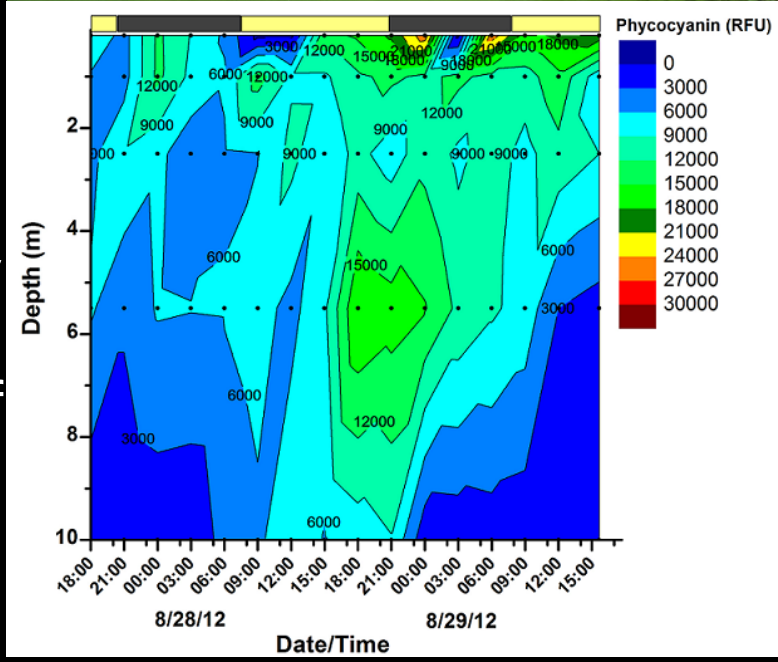
Definitive ID of toxin producers remains unresolved.

Authors suggest isolating and culturing specimens to determine their toxicity → instead, shotgun metagenomics could be used

Fetscher et al. 2015

Sample collection

- Collection approach varies by need
- Public health - collect scum from most impacted site
- Environmental - offshore, depth integrated (1-2 SD)
- Collect 0.5L in sterile glass or plastic bottles (PETG)
- Store on wet ice in the dark until processing
  - Do not freeze liquid samples if microscopy or DNA analysis is desired
- Ideally collect samples at same time of day for uniformity due to diel migration patterns



Diel migration - Copco Reservoir

Triggers for classifying cyanobacteria impaired waterbodies in California

Table 1. CyanoHAB Trigger Levels for Human Health

	Caution Action Trigger	Warning TIER I	Danger TIER II
Primary Triggers [a]			
Total Microcystins [b]	**0.8** µg/L	**6** µg/L	**20** µg/L
Anatoxin-a	**Detection** [c]	**20** µg/L	**90** µg/L
Cylindrospermopsin	**1** µg/L	**4** µg/L	**17** µg/L
Secondary Triggers			
Cell Density *(Toxin Producers)*	**4,000** cells/mL	--	--
Site Specific Indicators of Cyanobacteria	Blooms, scums, mats, ect.	--	--

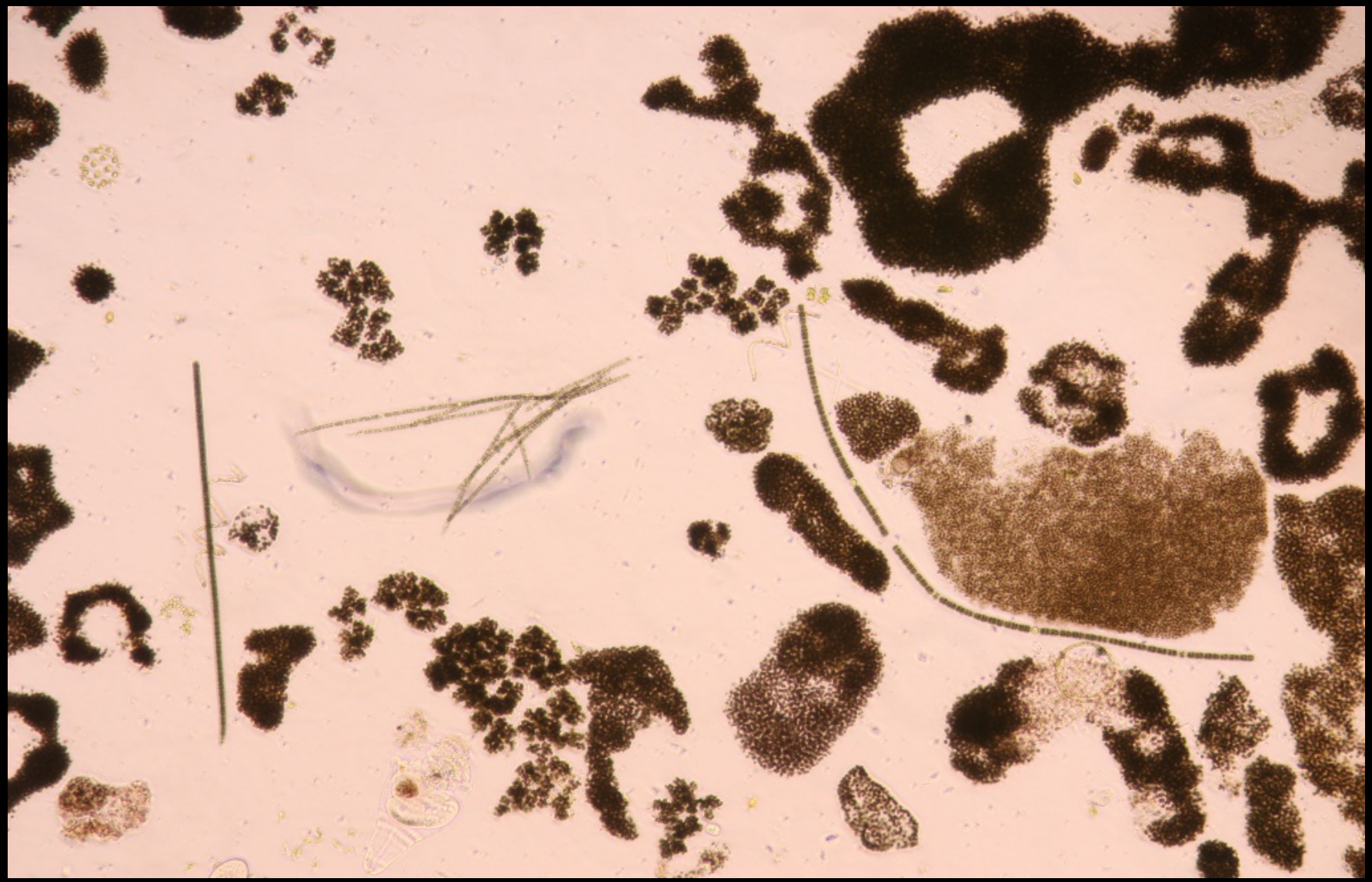
[a] The primary triggers are met when ANY toxin exceeds criteria.
[b] Microcystins refers to the sum of all measured microcystin variants. (See Box 3)
[c] Must use an analytical method that detects ≤ 1µg/L Anatoxin-a.

Saxitoxin should probably also be included

Potential toxins produced by common cyanobacterial genera

Cyanobacterial Genera	Anatoxin-a	Cylindrospermopsin	Microcystin	Nodularin	Saxitoxin
Aphanizomenon	X	X			X
Anabaena/Dolichospermum	X	X	X		X
Cylindrospermopsis	X	X			X
Fischerella			X		
Gloeotrichia			X		
Lyngbya					X
Microcystis			X		
Nodularia				X	
Nostoc			X		
Oscillatoria	X	X	X		
Phormidium	X				
Planktothrix	X		X		X
Pseudanabaena					
Raphidiopsis	X	X			X

Different CyanoHAB taxa present different cyanotoxin risks

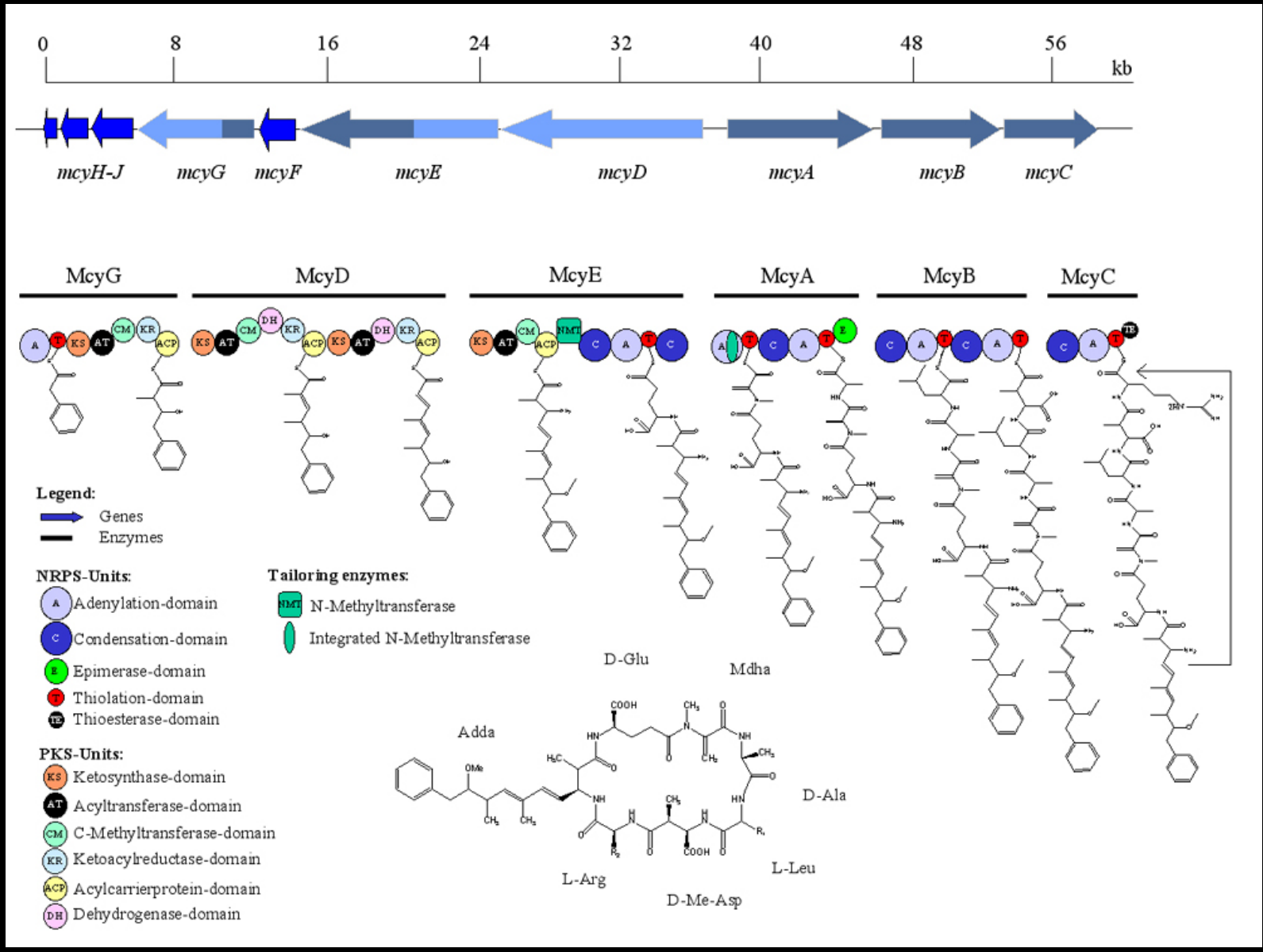


How does one best quantify health risks of transient blooms?

- Cells move vertically in water column and by wind/waves
- Population may be comprised of toxic and nontoxic strains
- Cells may produce varying amounts of toxin at different times

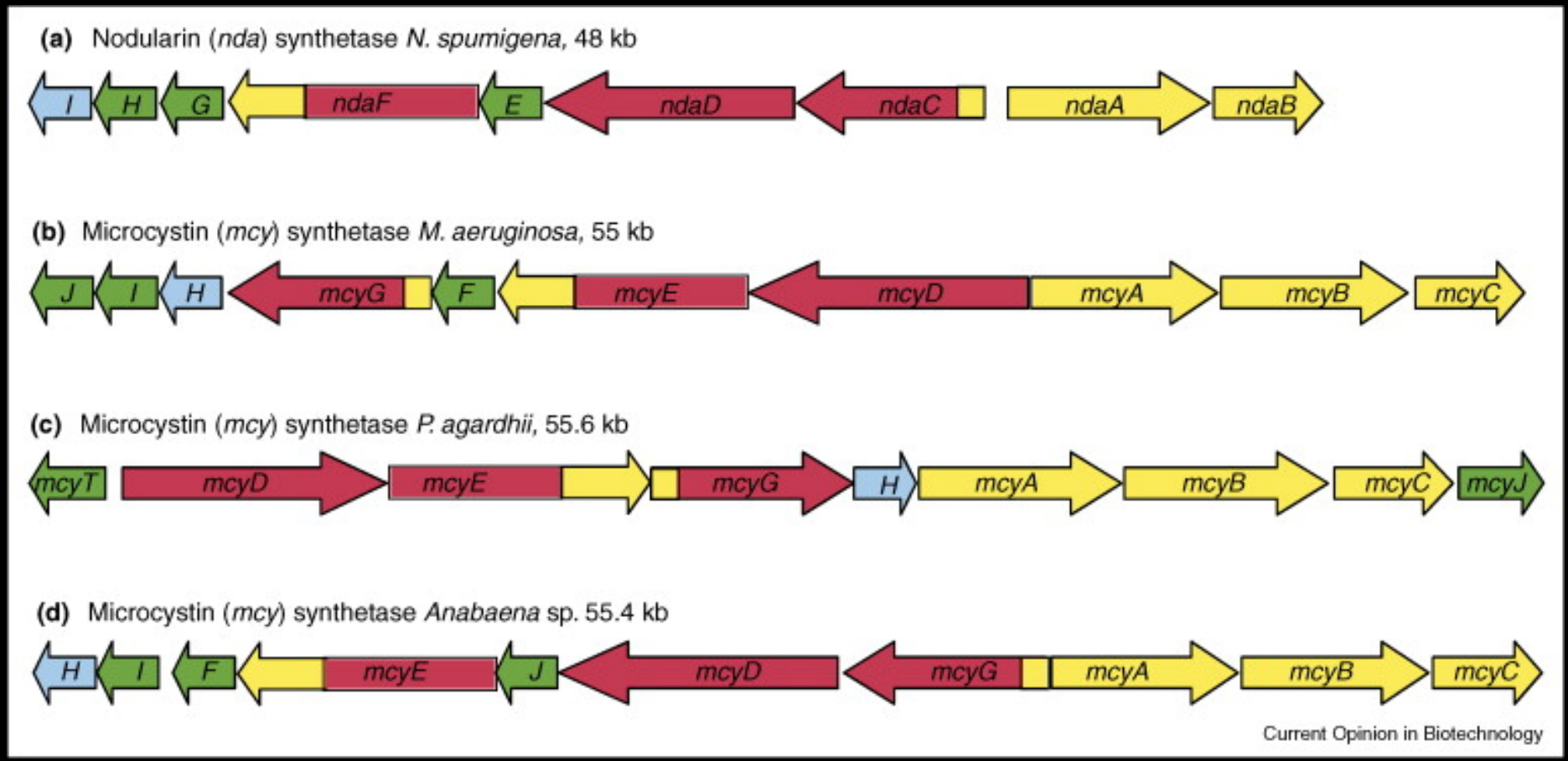
The key is to sample frequently and at many sites, but how can this be done cost effectively?

Biosynthetic assembly of microcystin



Dittmann et al., 2012

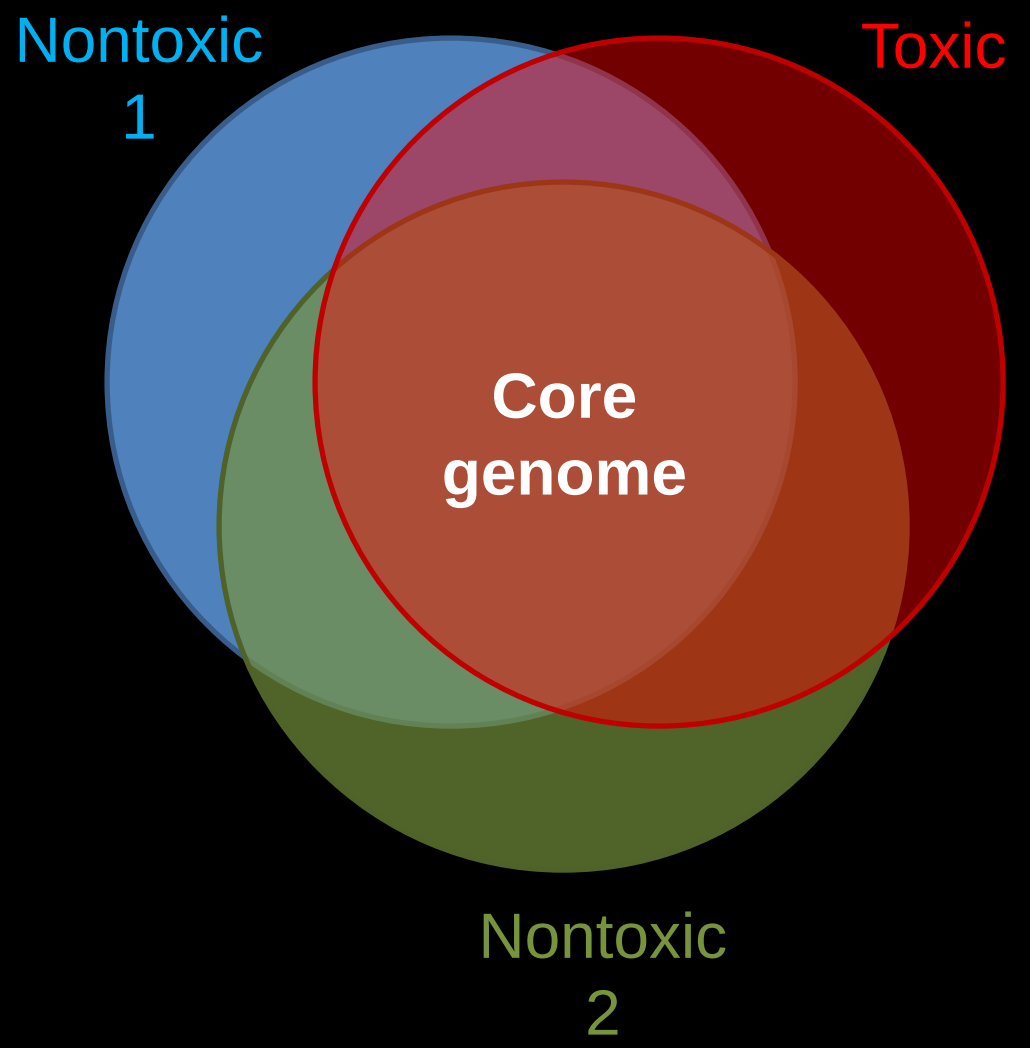
Structural organization of microcystin operon in diverse cyanobacteria

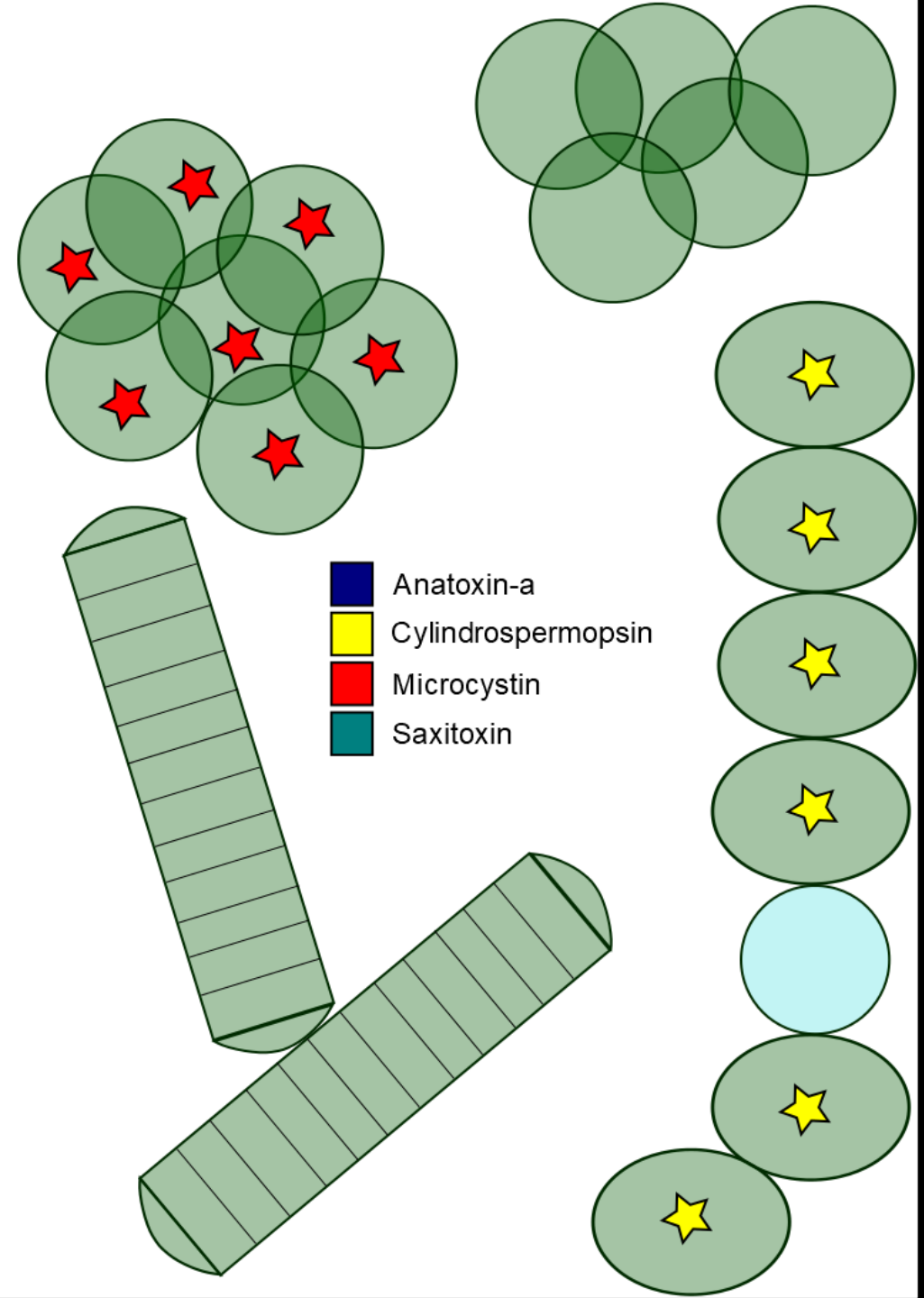


Pearson and Neilan, 2008

Why incorporate molecular tools into CyanoHAB monitoring?

- Rapid results
- Scalability
- High sensitivity
- High specificity
  - Target toxic cells
- Reproducible
- Non-subjective
- Lower cost
- Adaptable





QPCR “peers” into a cell’s genome

- Toxicity is strain-specific! Only cells with toxin genes can produce toxin
- Cells with toxin genes tend to use them (i.e., expression stays turned on)
- QPCR is used to quantify cyanotoxin genes
- Because the majority of toxin occurs intracellularly, gene abundance correlates well with toxin concentration

Received: 29 August 2017; Accepted: 4 October 2017; Published: 11 October 2017

marine drugs

MDPI

Article

The Abundance of Toxic Genotypes Is a Key Contributor to Anatoxin Variability in *Phormidium*-Dominated Benthic Mats

Susanna A. Wood [1,2] and Jonathan Puddick [1,*]

Abstract: The prevalence of benthic proliferations of the anatoxin-producing cyanobacterium *Phormidium* are increasing in cobble-bed rivers worldwide. Studies to date have shown high spatial and temporal variability in anatoxin concentrations among mats. In this study we determined anatoxin quotas (toxins per cell) in field samples and compared these results to the conventionally-used concentrations (assessed per dry weight of mat). Three mats were selected at sites in two rivers and were sampled every 2–3 h for 24–26 h. The samples were lyophilized and ground to a fine homogenous powder. Two aliquots of known weights were analyzed for anatoxin congeners using liquid chromatography-mass spectrometry, or digital droplet PCR with *Phormidium*-specific *anaC* primers to measure absolute quantities of gene copies. Anatoxin concentrations in the mats varied 59- and 303-fold in the two rivers over the study periods. A similar pattern was observed among gene copies (53- and 2828-fold). When converted to anatoxin quotas there was markedly less variability (42- and 16-fold), but significantly higher anatoxin quotas were observed in mats from the second river ($p < 0.001$, Student's *t*-test). There were no obvious temporal patterns with high and low anatoxin concentrations or quotas measured at each sampling time and across the study period. These results demonstrate that variability in anatoxin concentrations among mats is primarily due to the abundance of toxic genotypes. No consistent modulation in anatoxin production was observed during the study, although significant differences in anatoxin quotas among rivers suggest that site-specific physiochemical or biological factors may influence anatoxin production.

Keywords: benthic cyanobacteria; cobble-bed rivers; cyanotoxins; digital droplet PCR; liquid chromatography-mass spectrometry

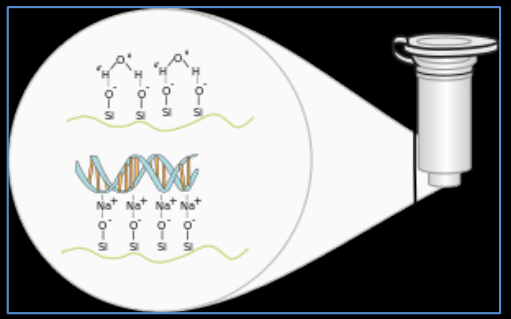
Sample processing

- Concentrate water samples by vacuum filtration (< 8 psi)
  - record filtration volume → essential for accurate gene estimates
  - Don’t freeze sample before filtering – it causes cell lysis
- Use sterile filtration rigs with glass fiber filters (0.7 or 1.2 um) or polyethersulfone (PES) membrane filters (0.2 or 0.45 um)
- Recommend 25 mm diameter instead of 47 mm filters
- Benthic scrapings can be centrifuged and decanted
- Store filters/pellets frozen (-20^{o}C good, -80^{o}C best)
  - Frozen filters can be archived for years

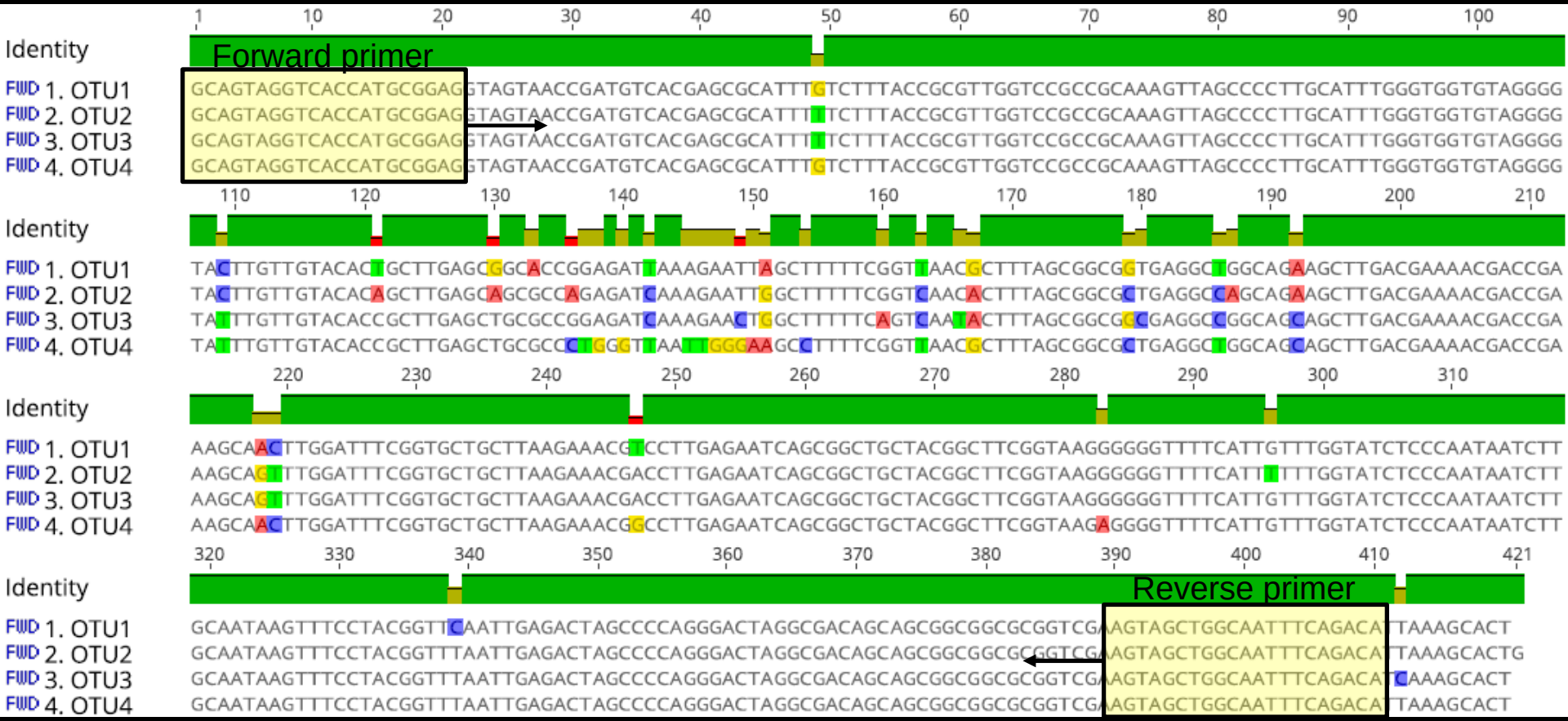
Concentrate cells

Lyse cells

Purify DNA



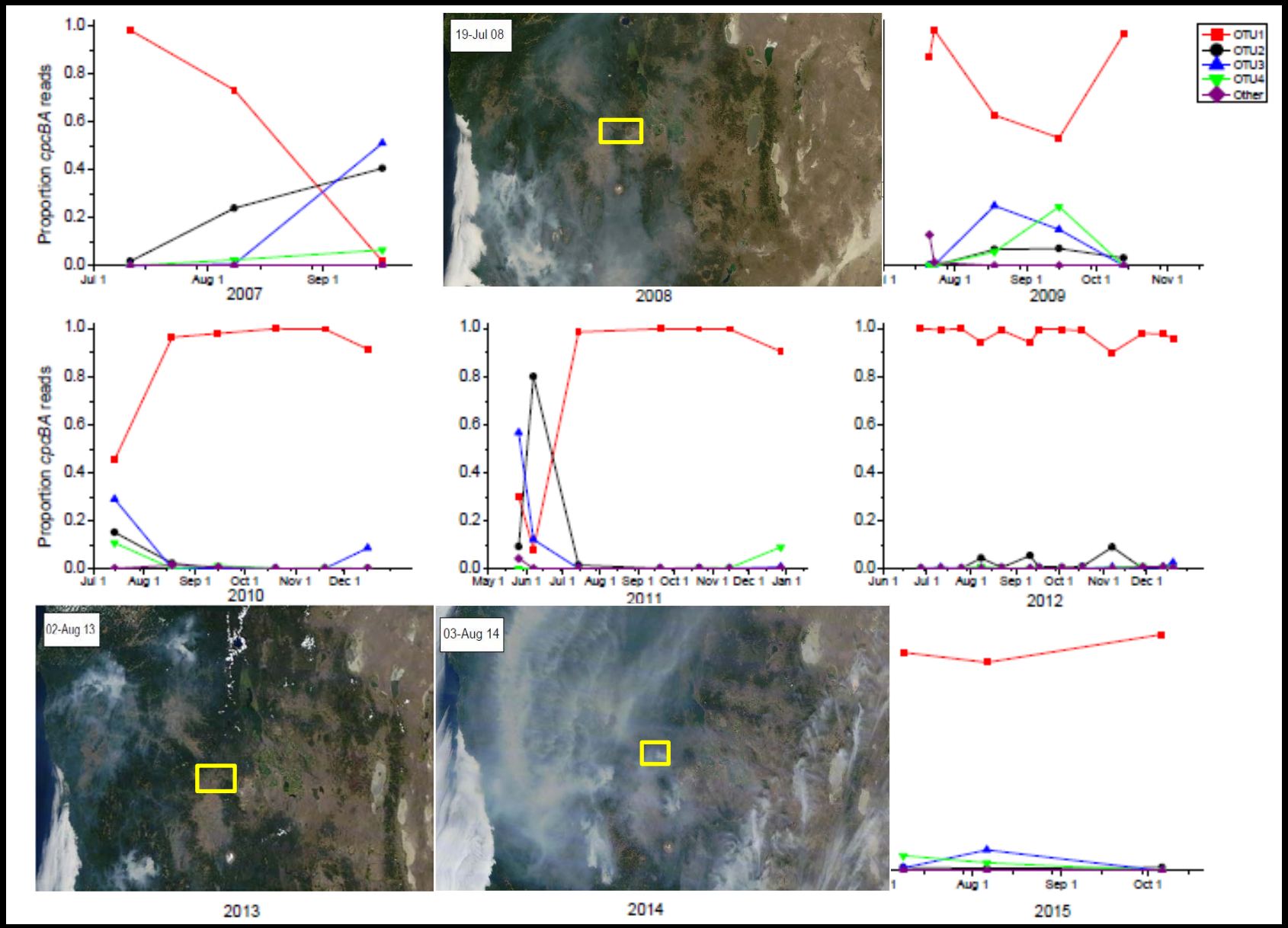
Design primers to match conserved regions of genes of interest, and if sequencing, choose sites that flank regions of heterogeneity



C-phycocyanin gene sequences from Klamath River *Microcystis* sp.

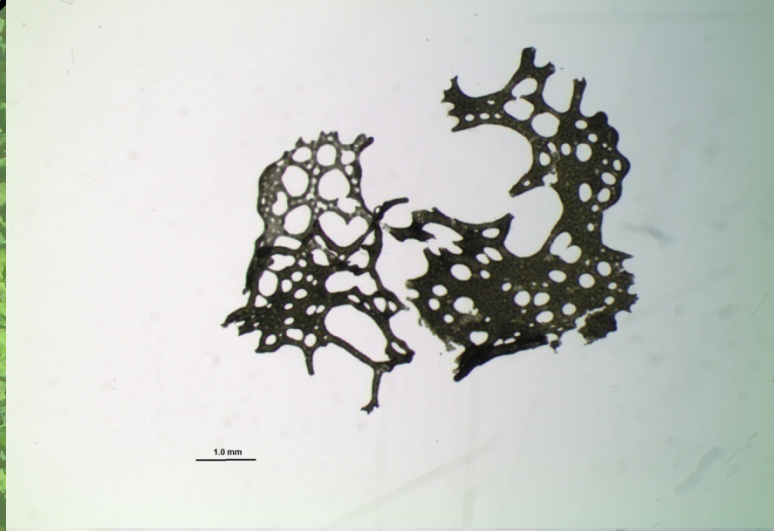
Otten et al., *In prep*

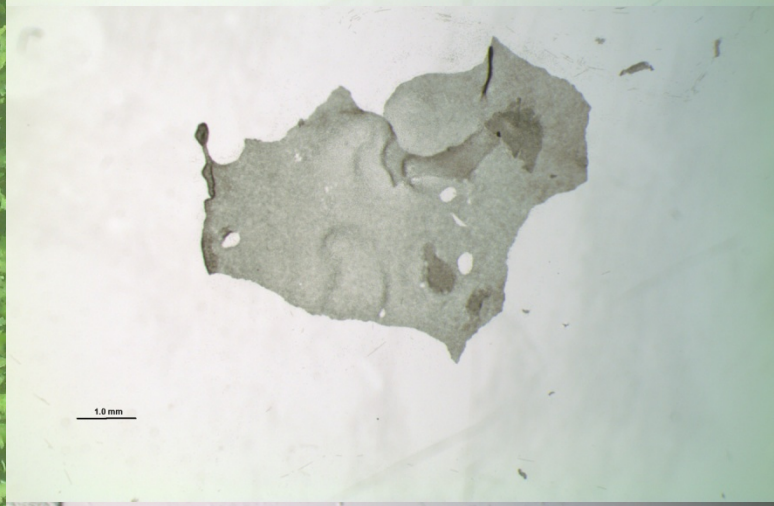
Microcystis strain diversity Copco Reservoir

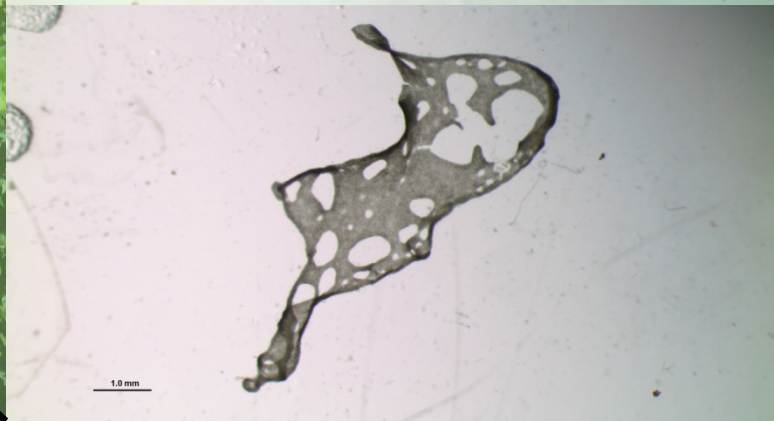


Otten & Dreher, *In prep.*

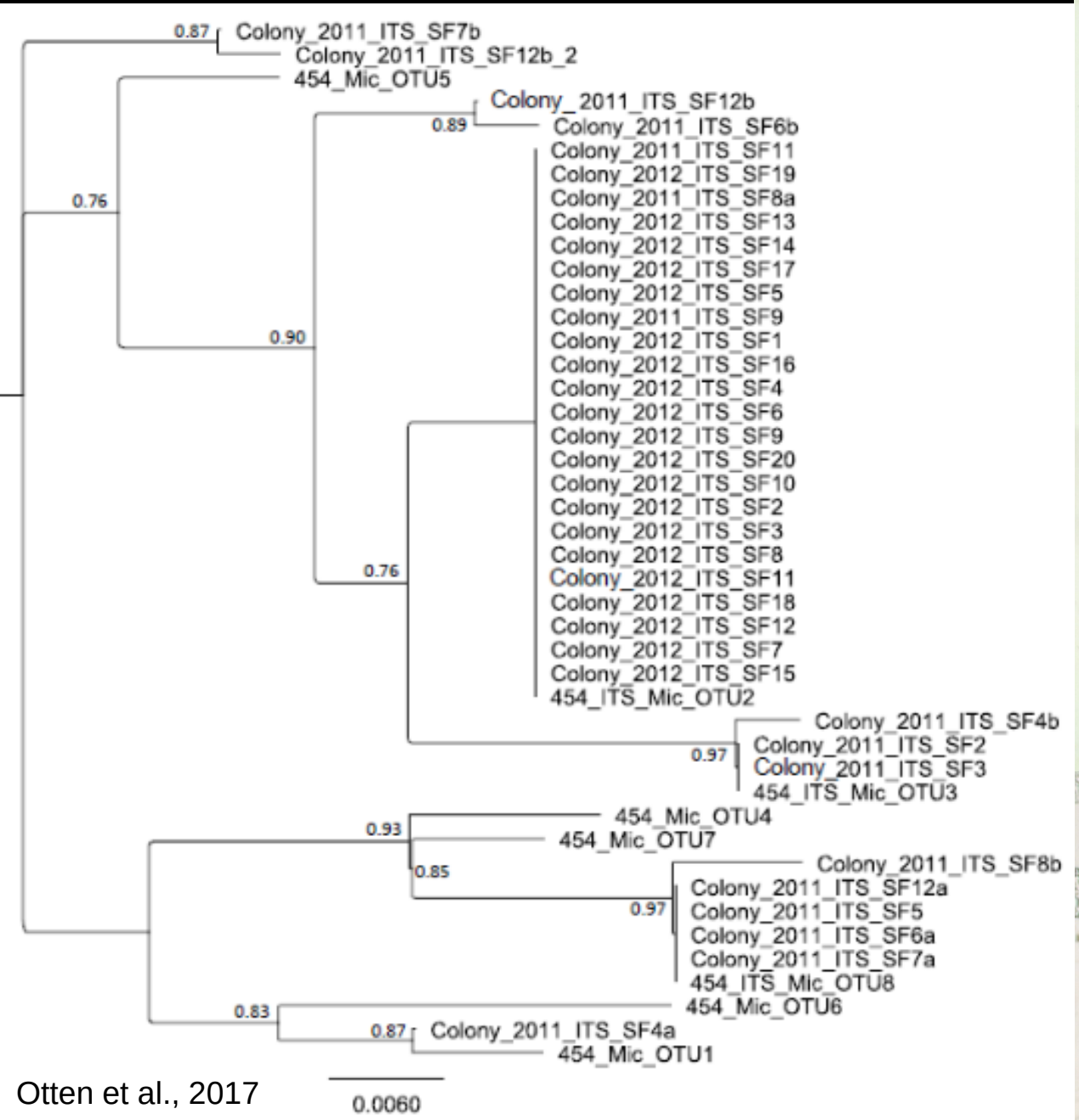
SF Delta – Mildred Island 2012



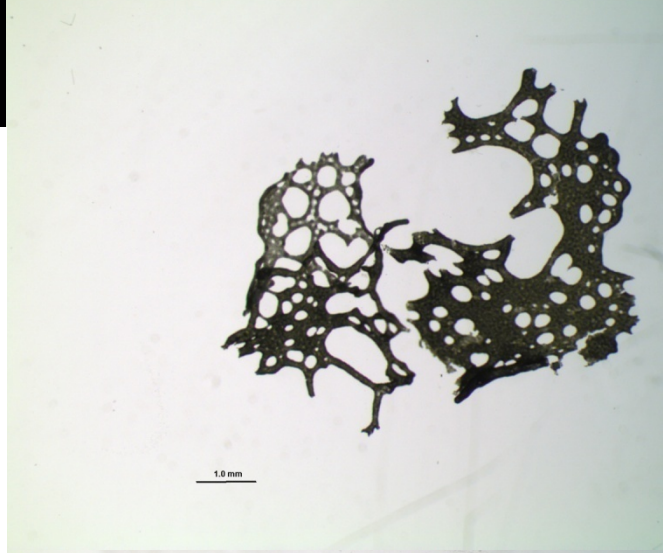


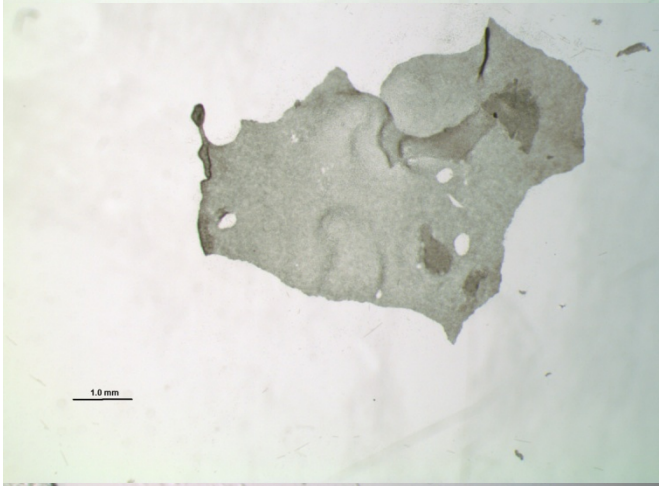


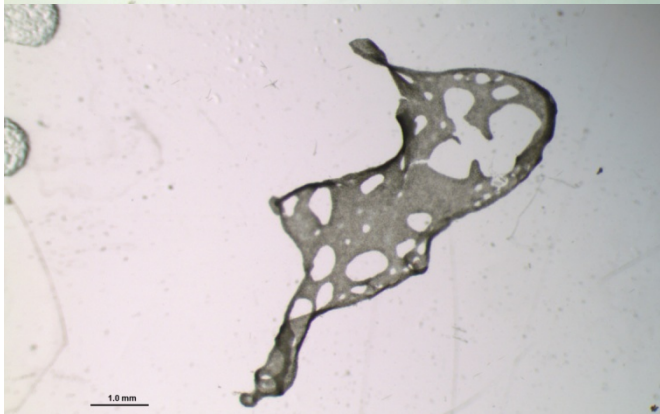
Phylogenetic Tree of SF Delta *Microcystis* sp.



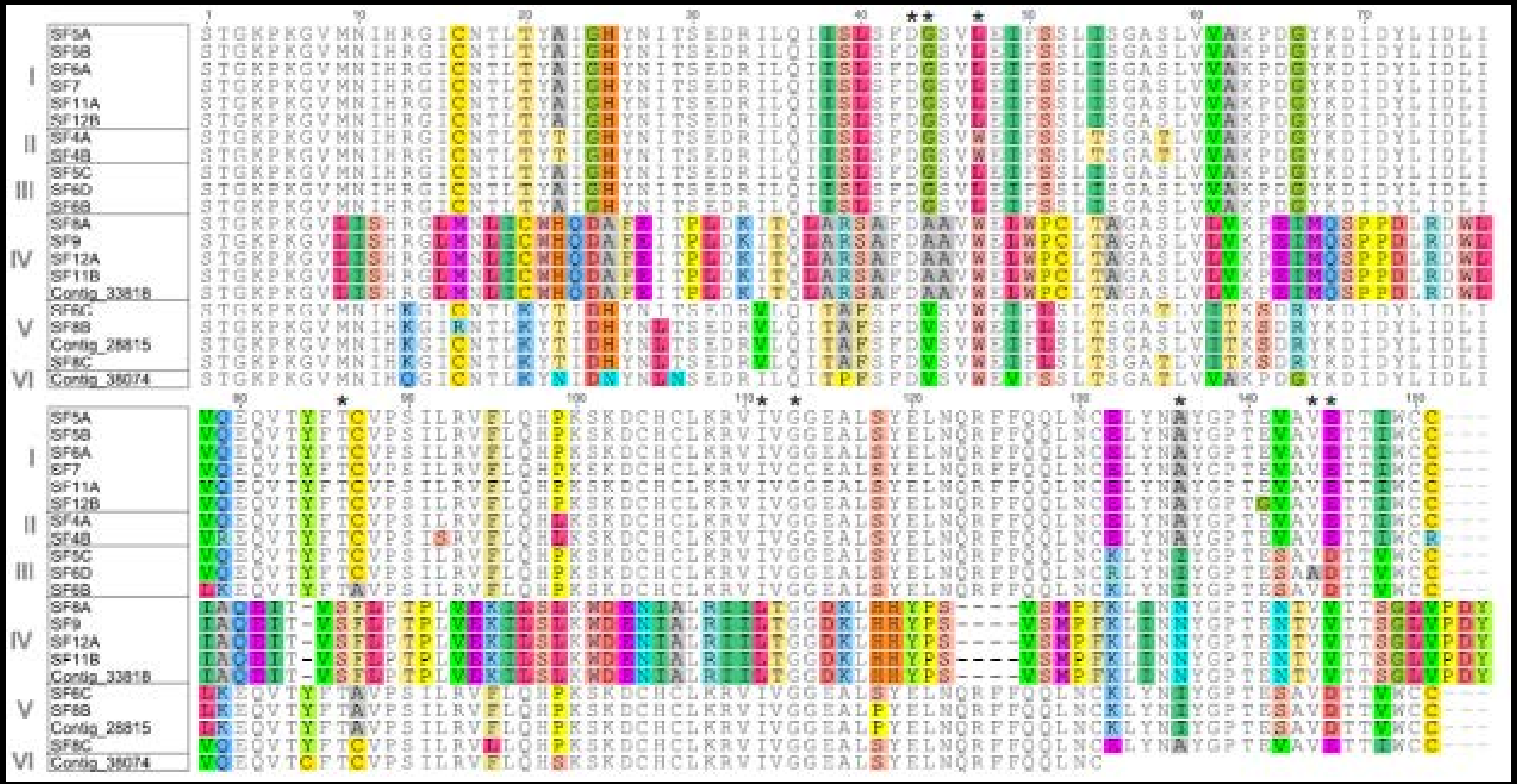
Otten et al., 2017







Evidence for at least six microcystin-producing strains in SF Delta



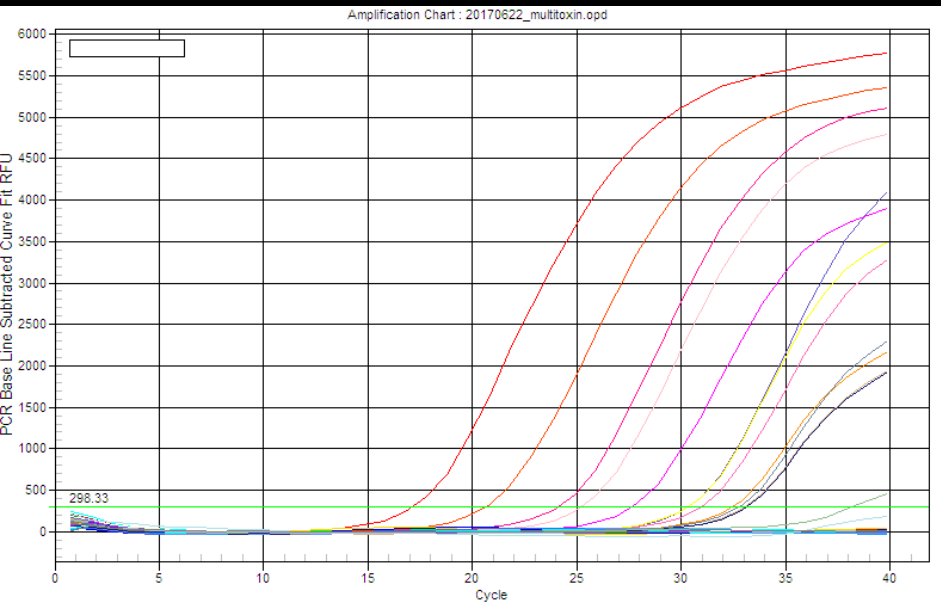
Amino acid translation of *mcyB* gene sequences

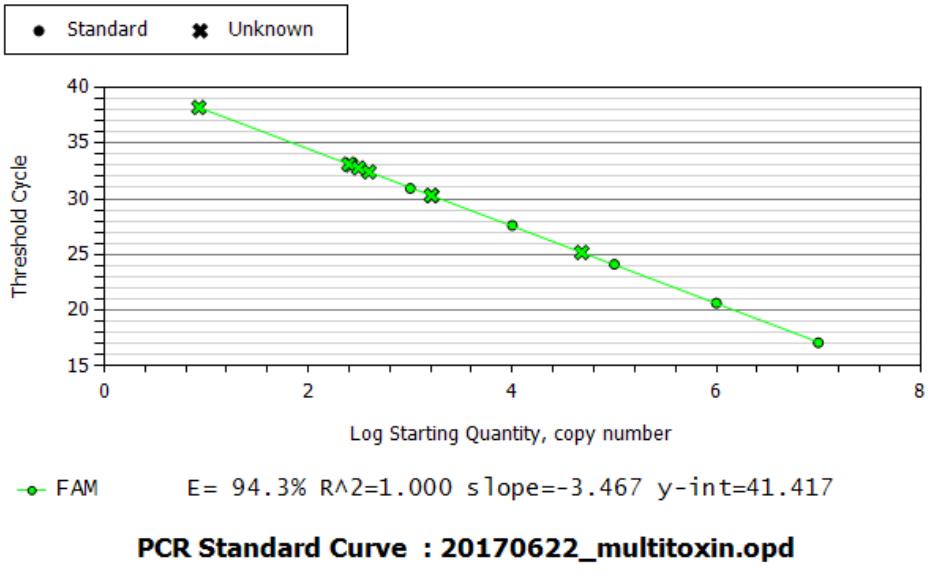
Otten et al., 2017

Overview of PCR-based tools

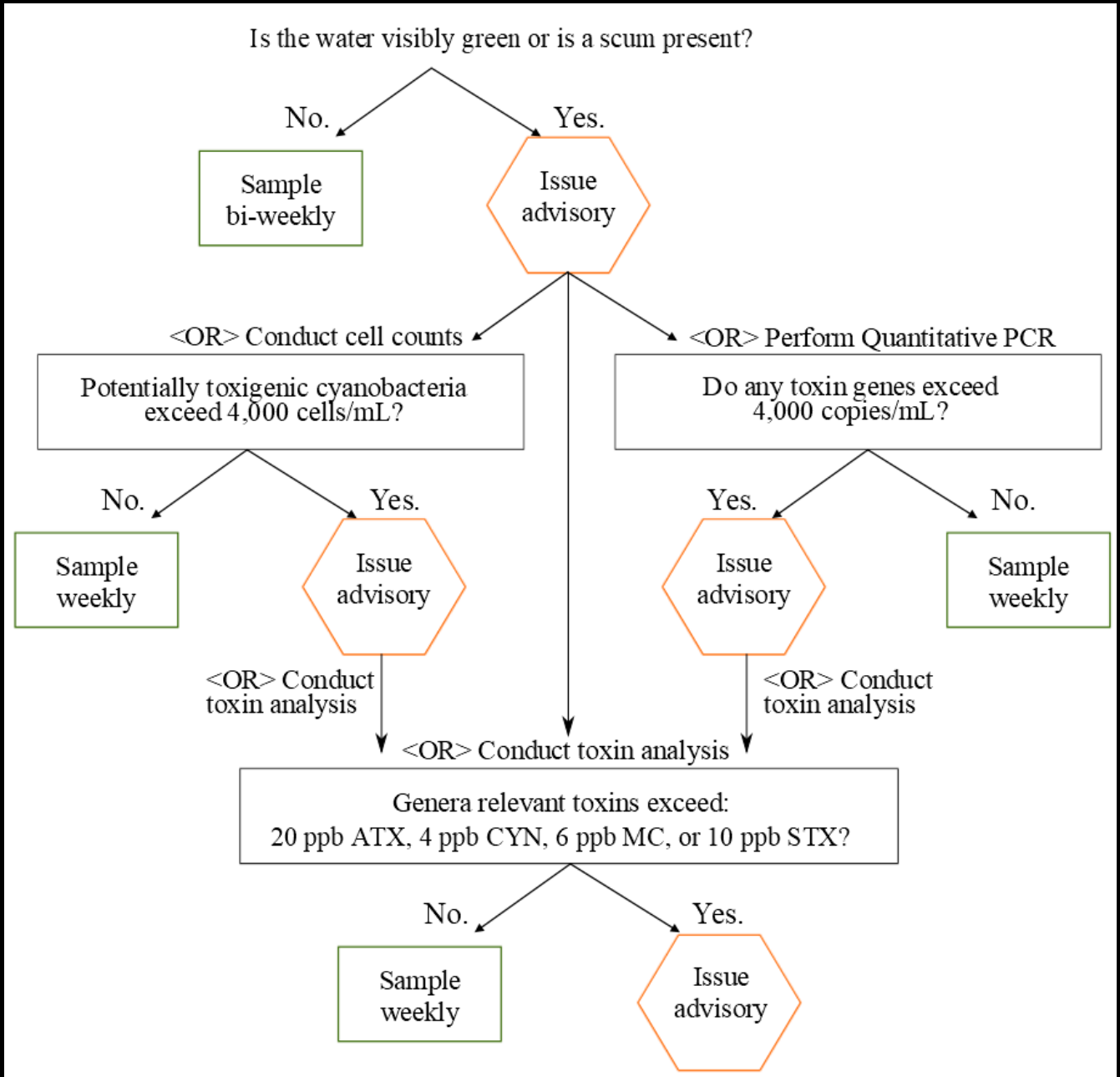
- Polymerase Chain Reaction (PCR) – the amplification of specific DNA sequences using complementary synthetic DNA molecules (primers)
  - Sequence information is required in order to design assays
  - Assays can be designed to be strain-specific or universal
  - DNA barcodes (index sequence tags) can be added to primers enabling multiple samples to be sequenced simultaneously (high throughput)

- Real-Time Quantitative PCR (QPCR) – same concept as regular PCR, but includes a fluorescent dye or probe allowing for absolute quantification of gene copies when related to a standard curve
  - Assumes gene copies/mL equivalent to cells/mL for single copy genes





QPCR as part of a tiered monitoring approach



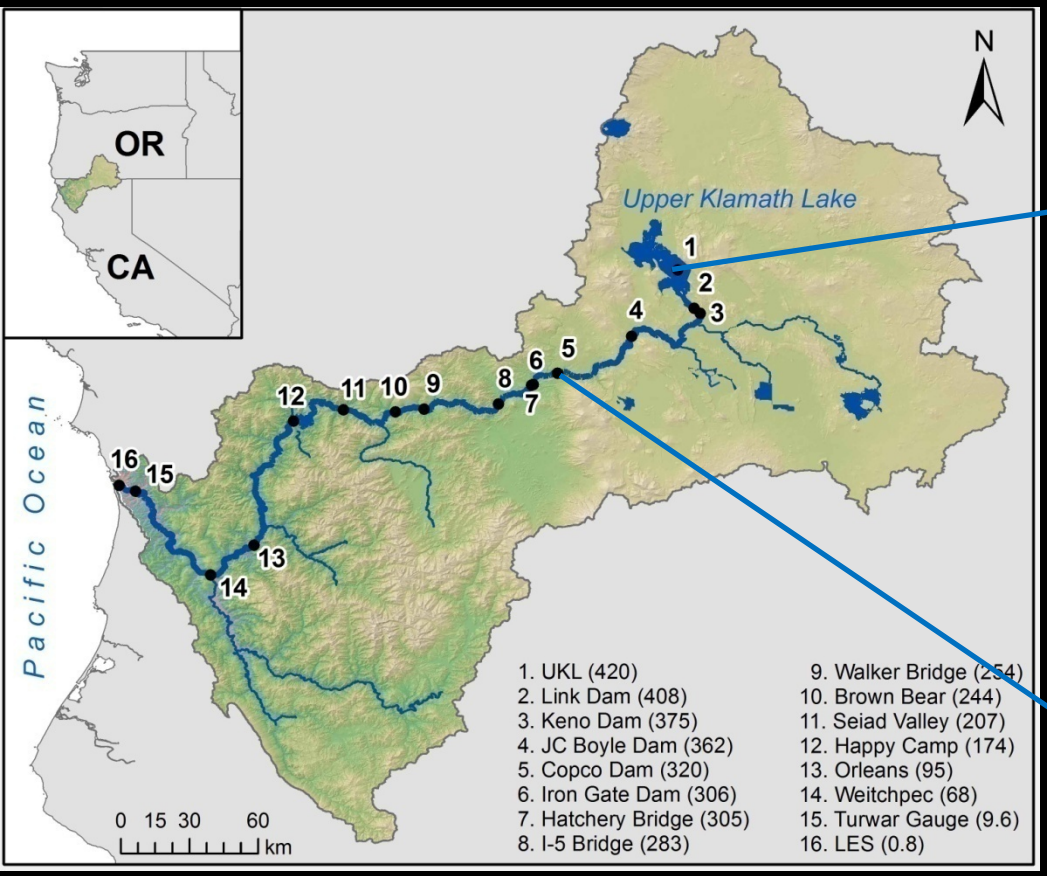
Ploidy in cyanobacteria complicates the relationship between cells and gene copies

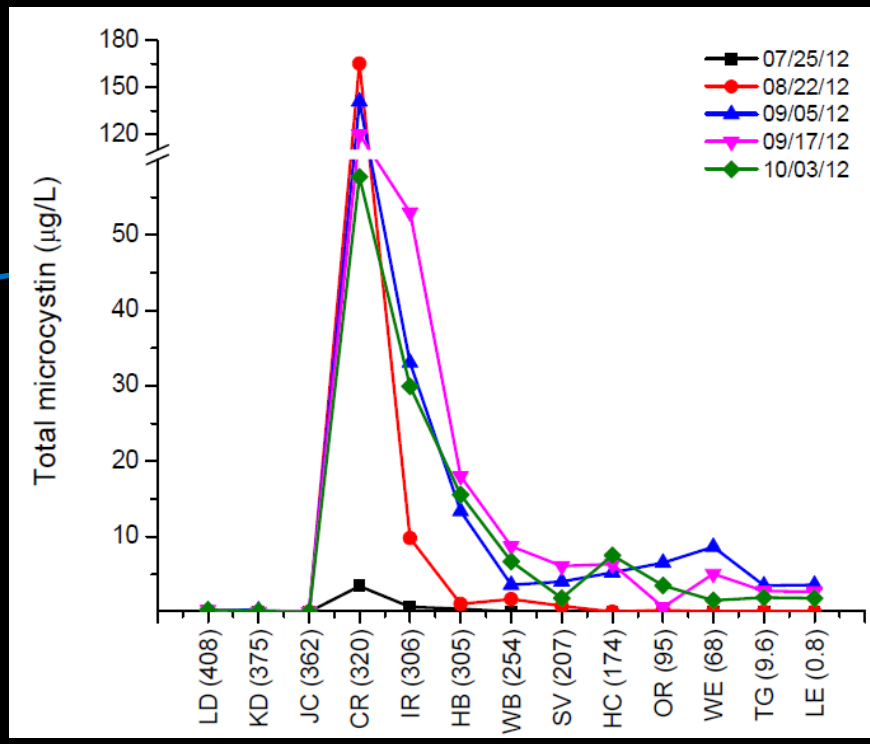
Table 3. Overview of cyanobacterial species with experimentally determined ploidy levels and selected parameters

Species	Growth temperature (°C)	Doubling time (h)	Genome size (Mbp)	Average genome copy No.	Ploidy	References
Anabaena cylindrica	30	18.5	–	25	Polyploid	Simon (1977)
Anabaena variabilis	30	–	7.1	5–8	Oligoploid	Simon (1980)
10 *Microcystis* strains	20	stat.ph.	–	1–10	Oligoploid	Kurmayer & Kutzenberger (2003)
Anabaena sp. PCC 7120	28	–	7.2	8.2	Oligoploid	Hu *et al.* (2007)
Prochlorococcus	–	–	1.7	–	Monoploid	Vaulot *et al.* (1995)
Synechococcus elongatus PCC 7942	28	24	2.8	3.9/3.3*	Oligoploid	This study
S. elongatus PCC 7942	30	11, LDC	2.8	3–5	Oligoploid	Mori *et al.* (1996)
Synechococcus sp. PCC 6301	38	5 to >50	2.7	2–6 to >1–2†	Oligoploid	Binder & Chisholm (1990)
Synechococcus sp. WH 7803	28	–	2.4	3.6	Oligoploid	This study
Synechococcus sp. WH 7803	25	–	2.4	2–4	Oligoploid	Binder & Chisholm (1995)
Synechococcus sp. WH 7805	25	15	2.6‡	1	Monoploid	Binder & Chisholm (1995)
Synechococcus sp. WH 8101	25	17	3.2‡	1	Monoploid	Armbrust *et al.* (1989)
Synechococcus sp. WH8103	25	22	2.7‡	1–2	Monoploid	Binder & Chisholm (1995)
Synechocystis sp. PCC 6803 (motile)	28	20	3.6	218/58/58*	Polyploid	This study
Synechocystis sp. PCC 6803 (GT)	28	20	3.6	142/47/43*	Polyploid	This study
Synechocystis sp. PCC 6803 ('Kazusa')	30	15–20	3.6	12	Polyploid	Labarre *et al.* (1989)

Griese et al., 2011

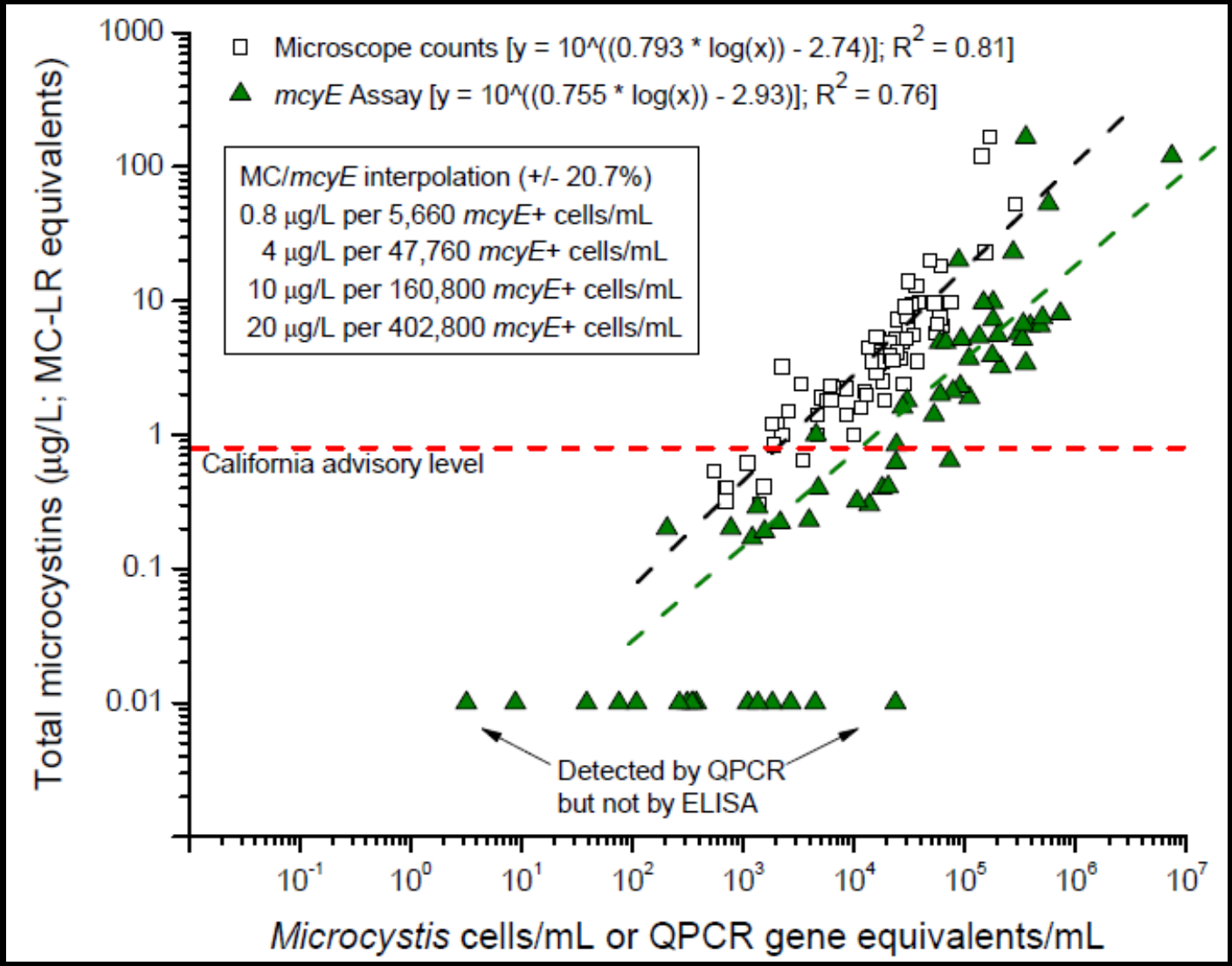
Molecular investigations of toxin-producing cyanobacteria in the Klamath River Estuary





Otten et al., 2015

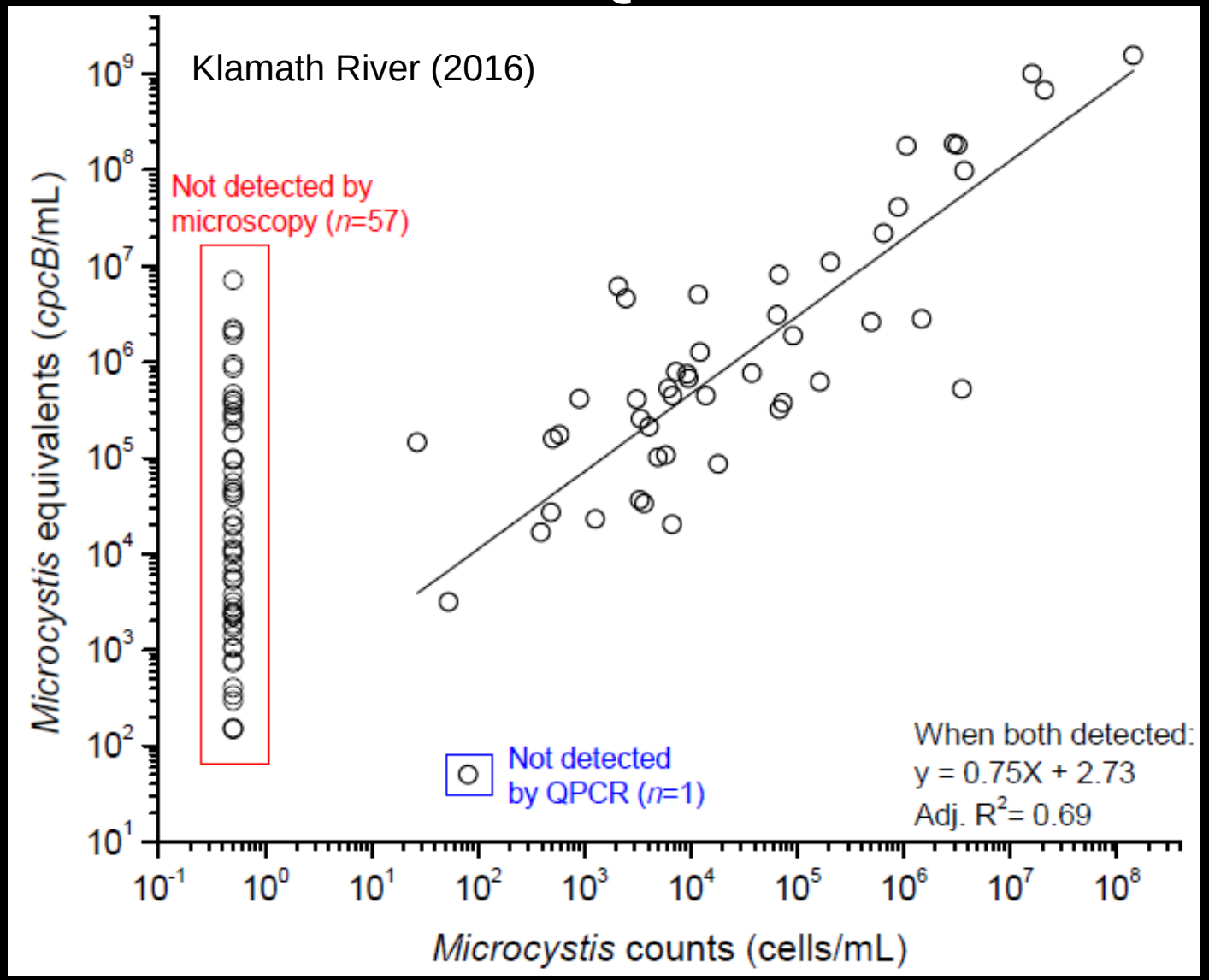
Comparison of methods - Microcystins vs QPCR (*mcyE*) estimates



All samples were 0.5 m grab samples

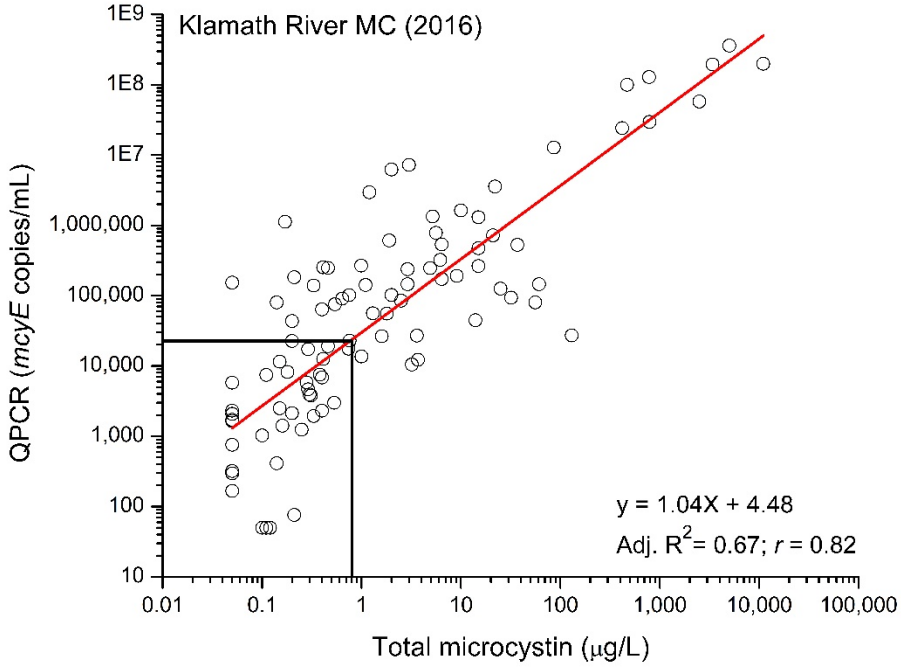
Otten et al., 2015

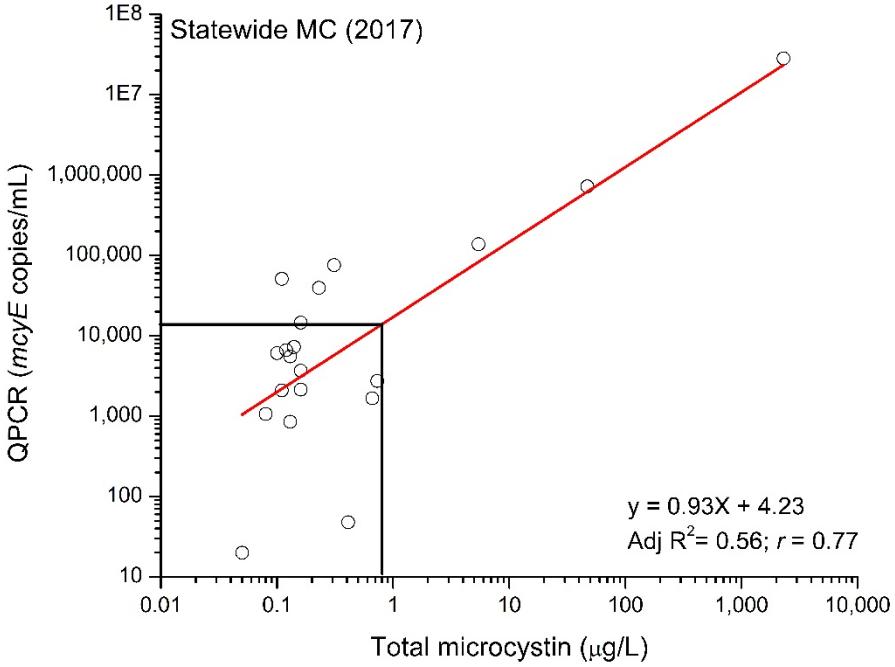
Comparison of methods - *Microcystis* cell counts vs QPCR estimates

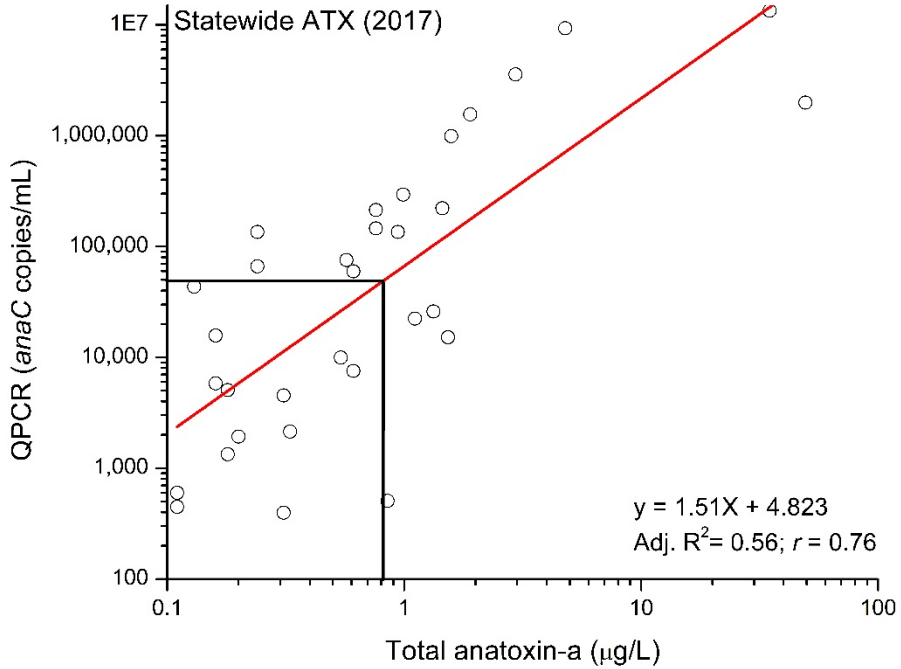


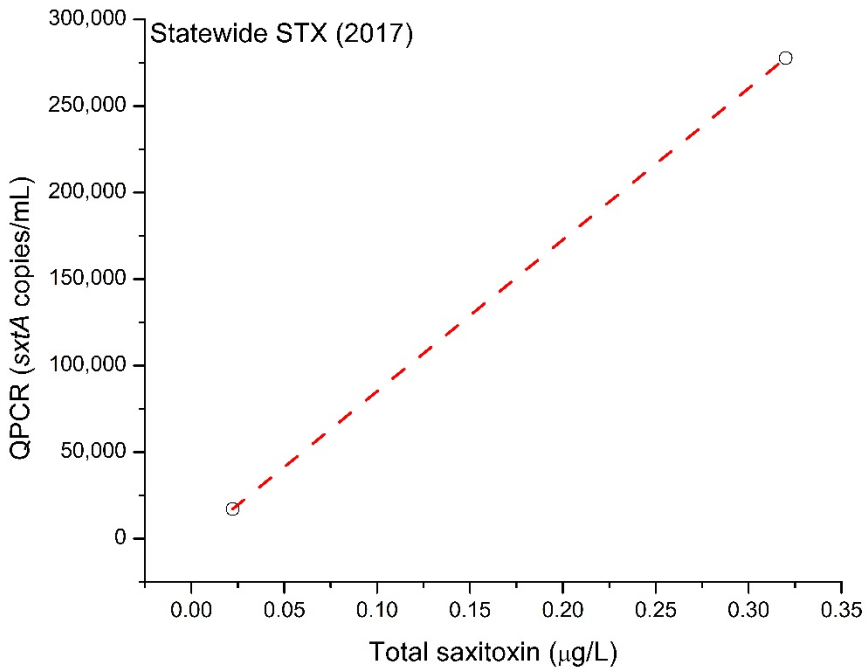
DNA $t_{1/2}$ in surface water is ~6 hours (Maruyama et al., 2014)

Otten, *in prep*.


Klamath River MC (2016)
QPCR (mcyE copies/mL)
Total microcystin (µg/L)
y = 1.04X + 4.48
Adj. R2= 0.67; r = 0.82


Statewide MC (2017)
QPCR (mcyE copies/mL)
Total microcystin (µg/L)
y = 0.93X + 4.23
Adj R2= 0.56; r = 0.77


Statewide ATX (2017)
QPCR (anaC copies/mL)
Total anatoxin-a (µg/L)
y = 1.51X + 4.823
Adj. R2= 0.56; r = 0.76

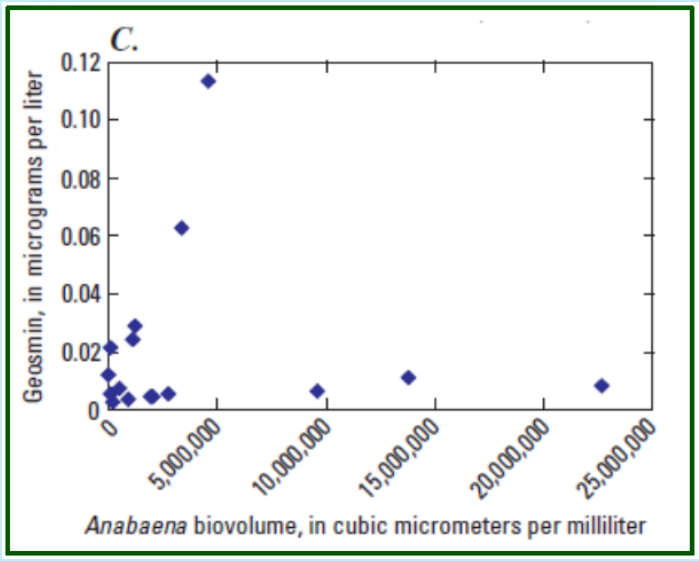

Statewide STX (2017)
QPCR (sxtA copies/mL)
Total saxitoxin (µg/L)

Long-term investigation of T&O issues in Cheney Reservoir (Kansas)

USGS
science for a changing world

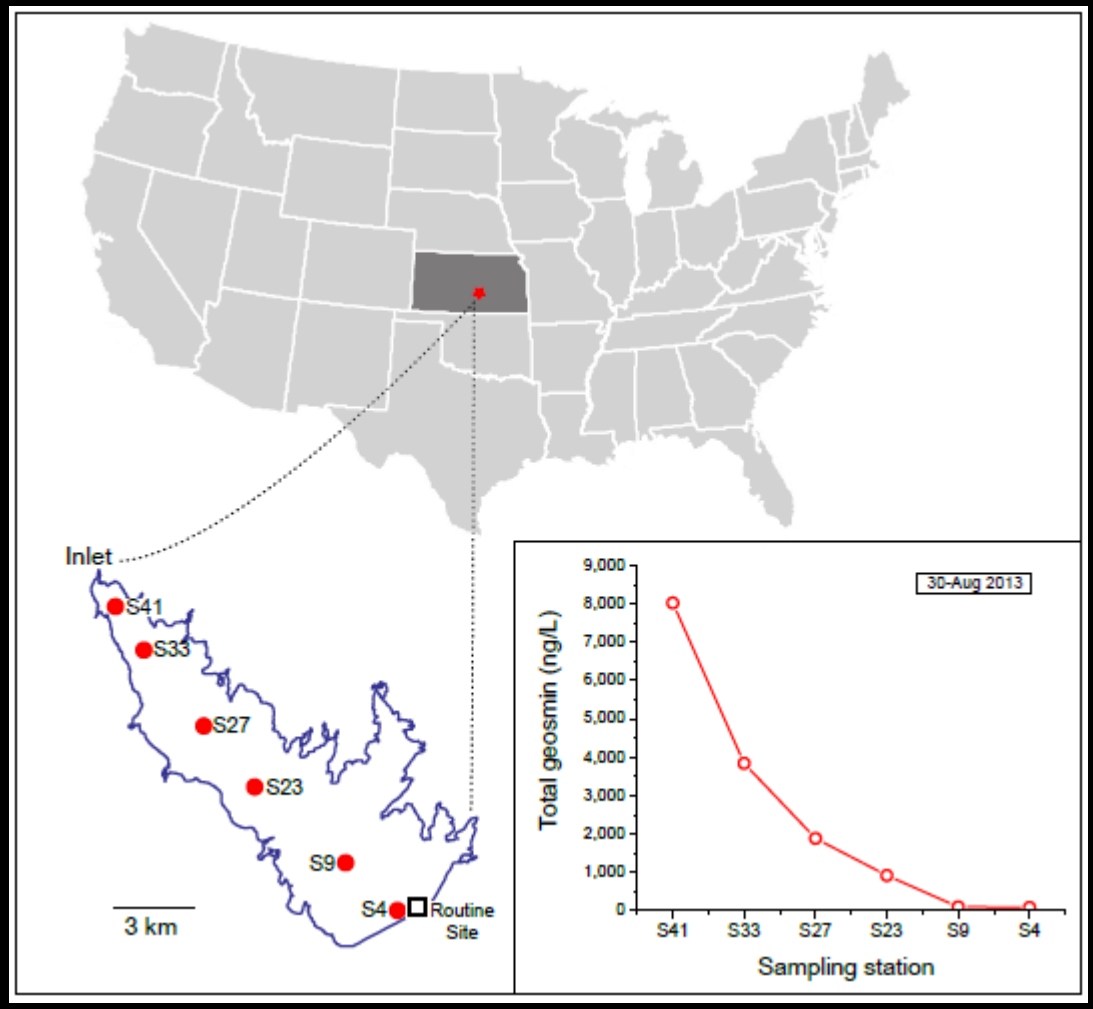
Prepared in cooperation with the
City of Wichita, Kansas

Water Quality and Relation to Taste-and-Odor Compounds in the North Fork Ninnescah River and Cheney Reservoir, South-Central Kansas, 1997–2003



U.S. Department of the Interior
U.S. Geological Survey

Christensen et al., 2006

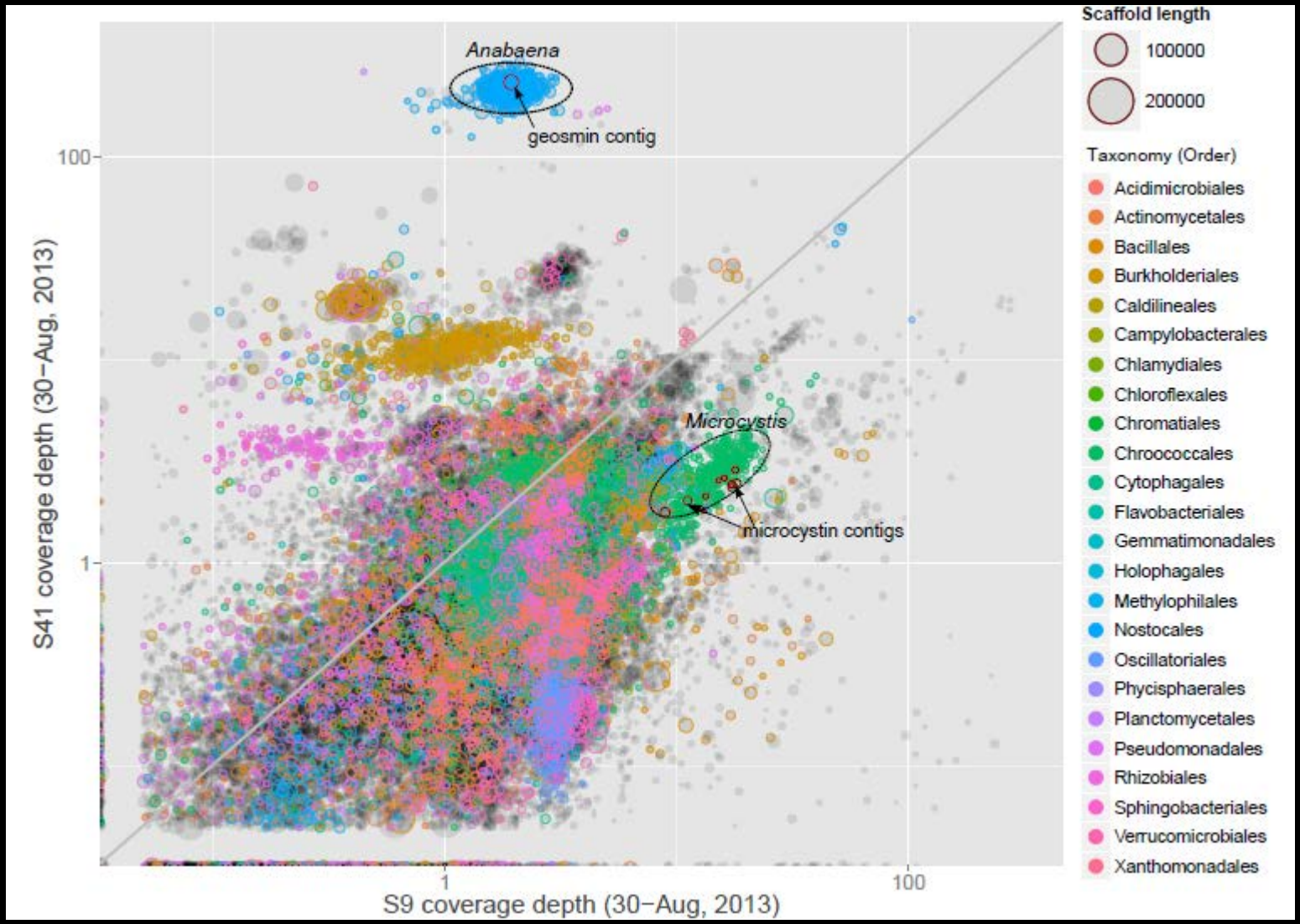


Otten et al., 2016

Bioinformatic Workflow

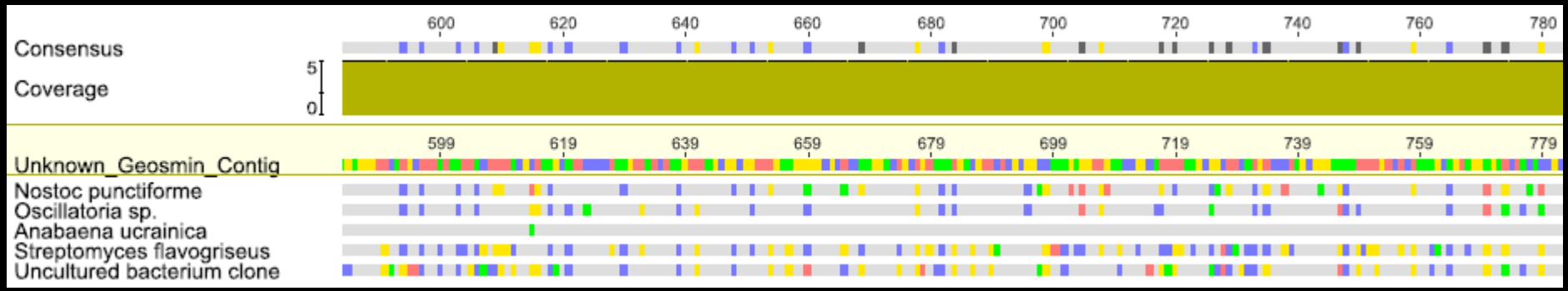
1. Sequence DNA (Nextera XT kits + Illumina HiSeq 2000) = ~270 million reads/
2. Concatenate reads and assemble contigs (IDBA_UD)
3. Calculate contig coverage depths for each sample (BWA / SAMtools)
4. Taxonomically classify/bin contigs (BLAST, MEGAN, mmgenome, Phylopythia
5. Read data into R and analyze

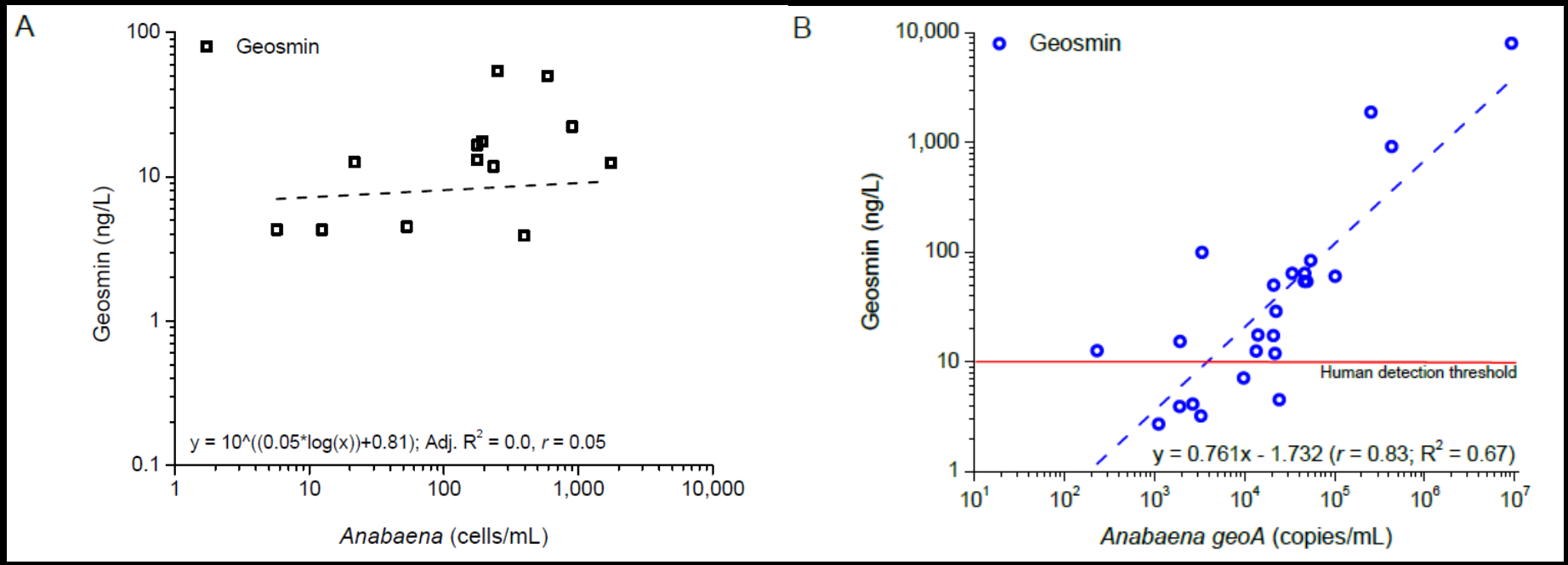
Identification of toxin and T&O producing bacteria by shotgun metagenomics



Otten et al., 2016

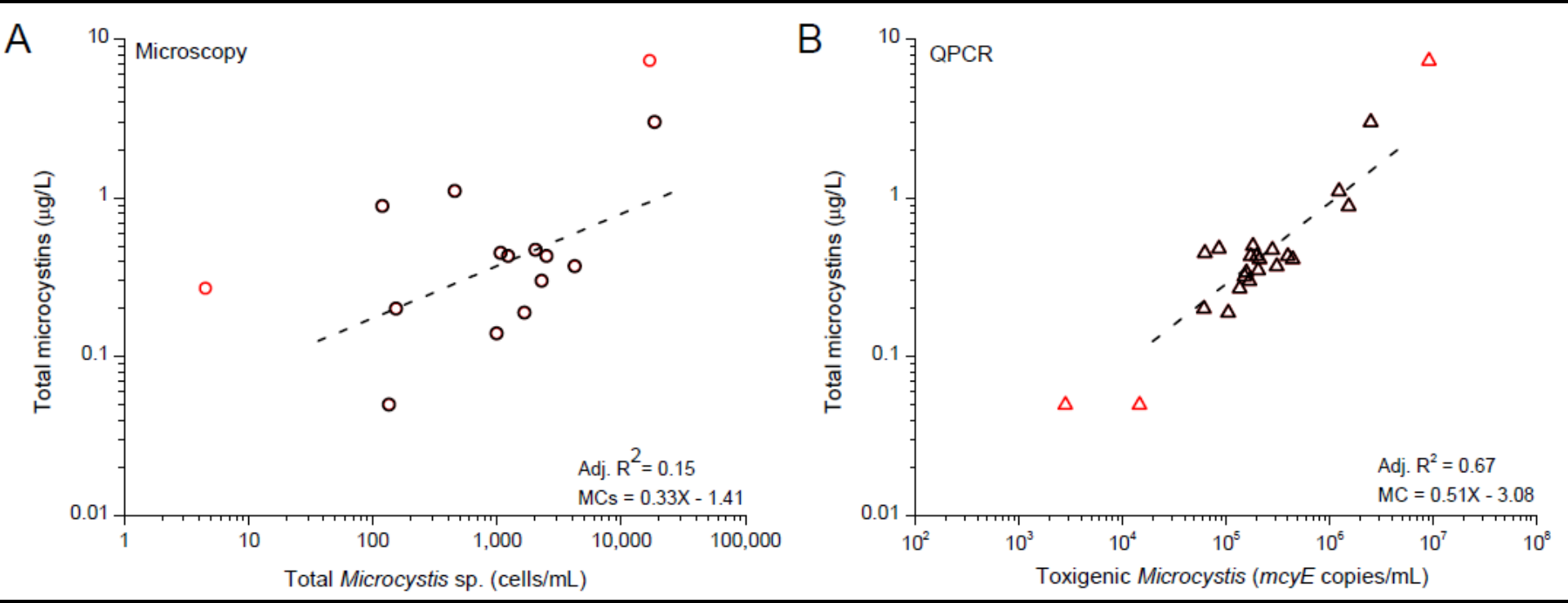
Development of a QPCR assay for geosmin





Otten et al., 2016

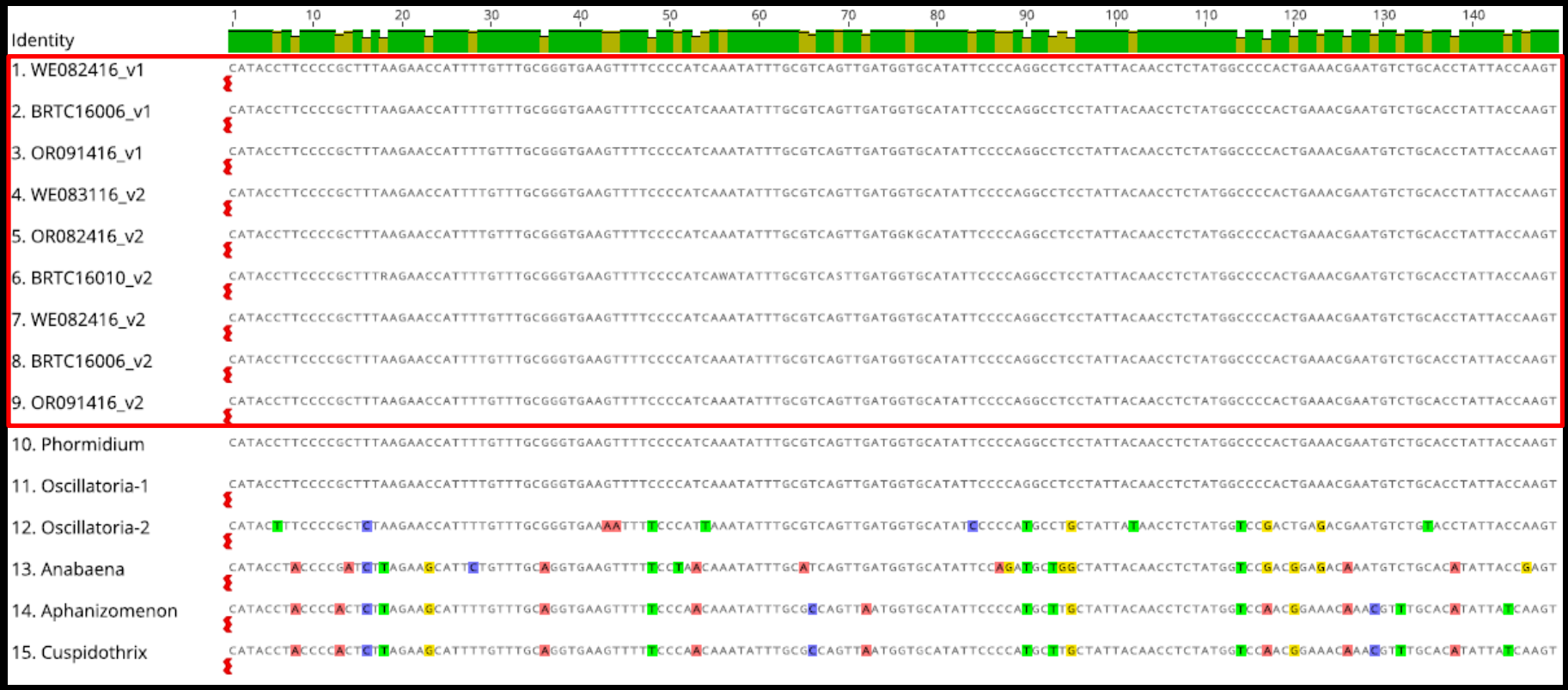
Comparison of QPCR & microscopy for estimating microcystin in Cheney Reservoir



Otten et al., 2016

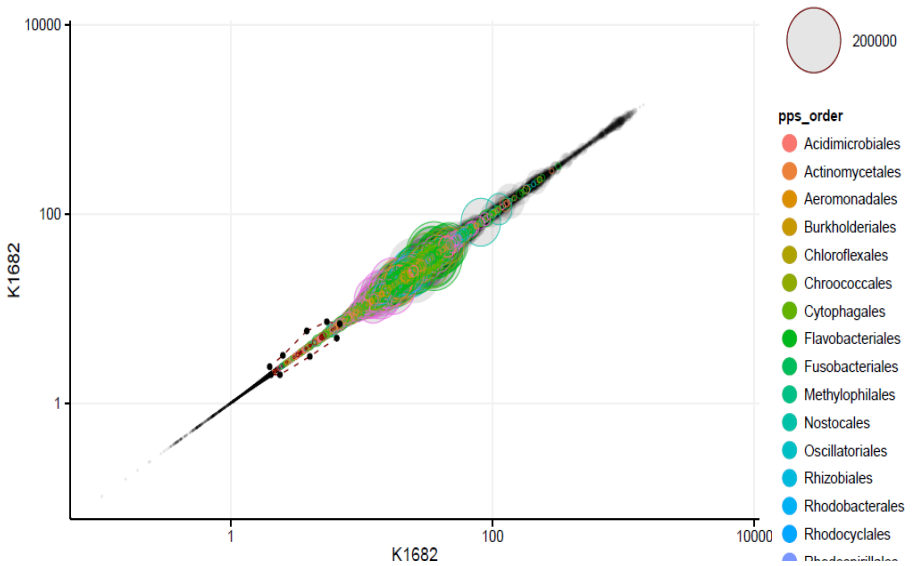
DNA source-tracking enabled ID of and ATX-producing cyano in the Klamath River

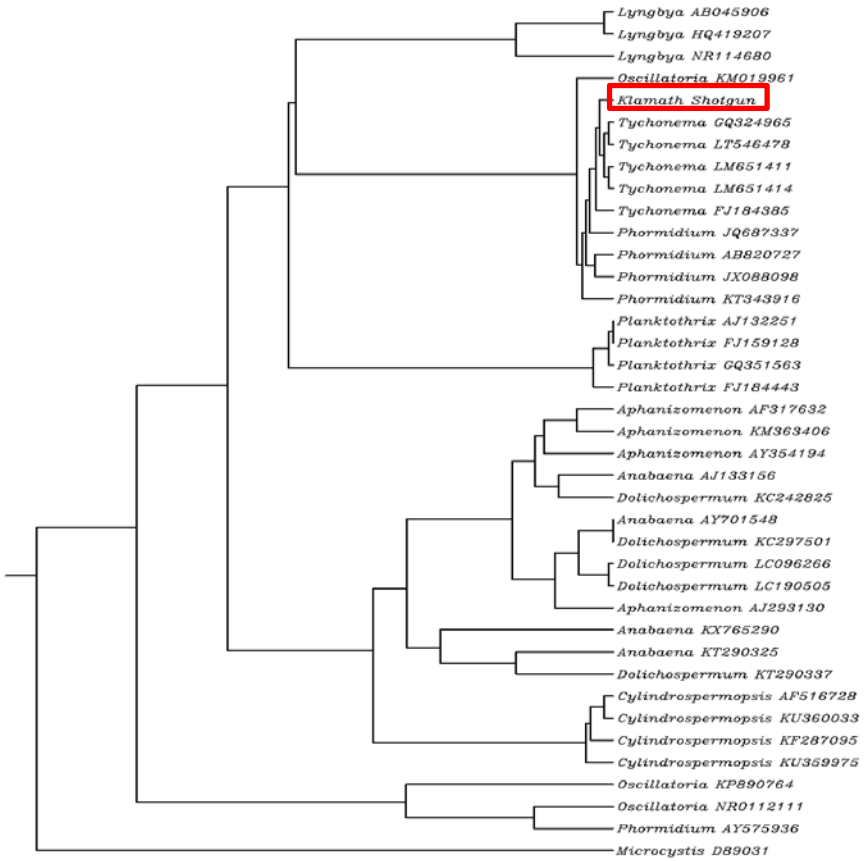
- Screened for ATX genes in routine water column grab samples (*n*=128) in 2016
- 32% of samples contained ATX genes above the LOQ (100 gene copies/mL)
- Strongest hits were confirmed by Sanger Sequencing.
- From these results three samples were selected for shotgun sequencing.



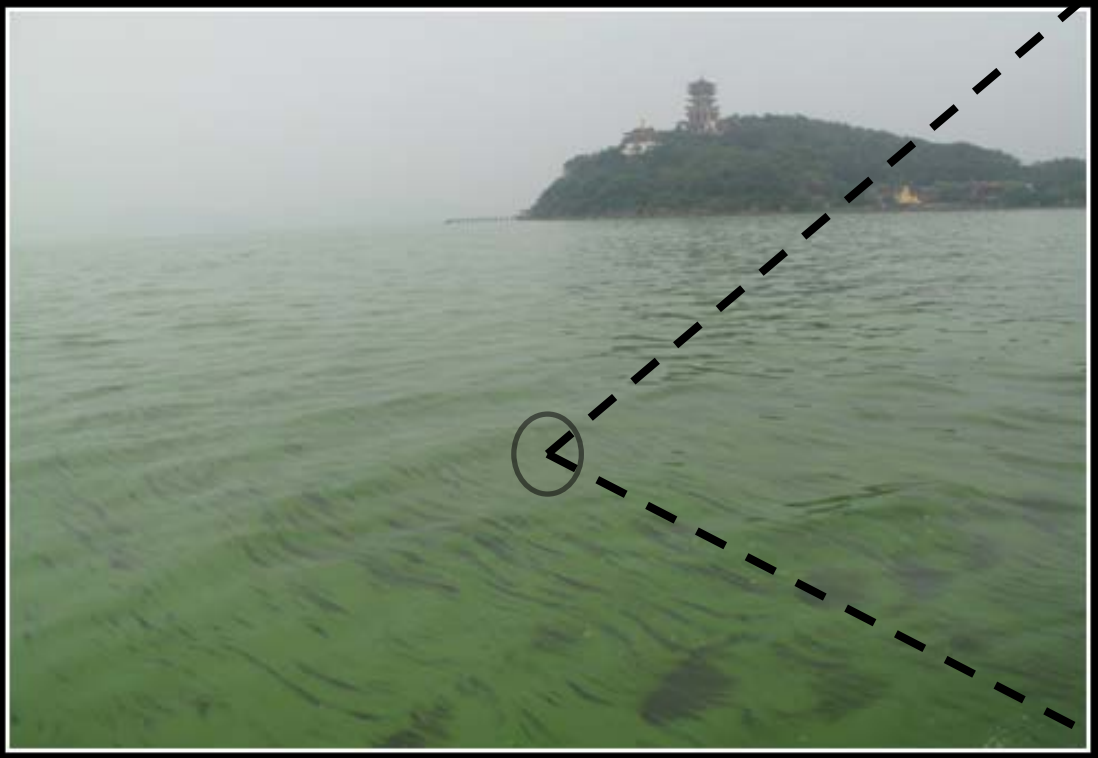
Sequencing results

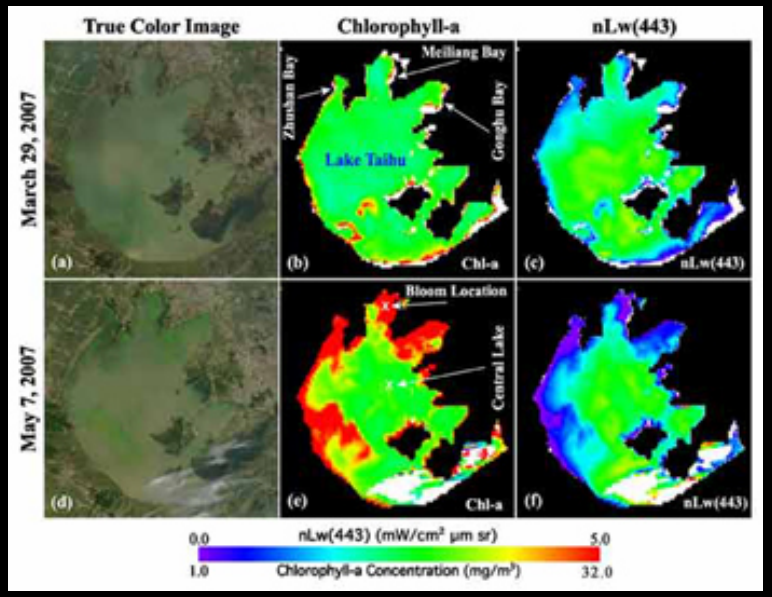
- Assembled ~18,000 bp of the ATX operon
- Nucleotide similarity was only ~90% with other published sequences
- Recovered a 16S rRNA sequence within the ATX genome bin
- The 16S rRNA sequence indicates that the ATX producer is most likely *Phormidium* sp. or *Tychonema* sp., and therefore of benthic origin





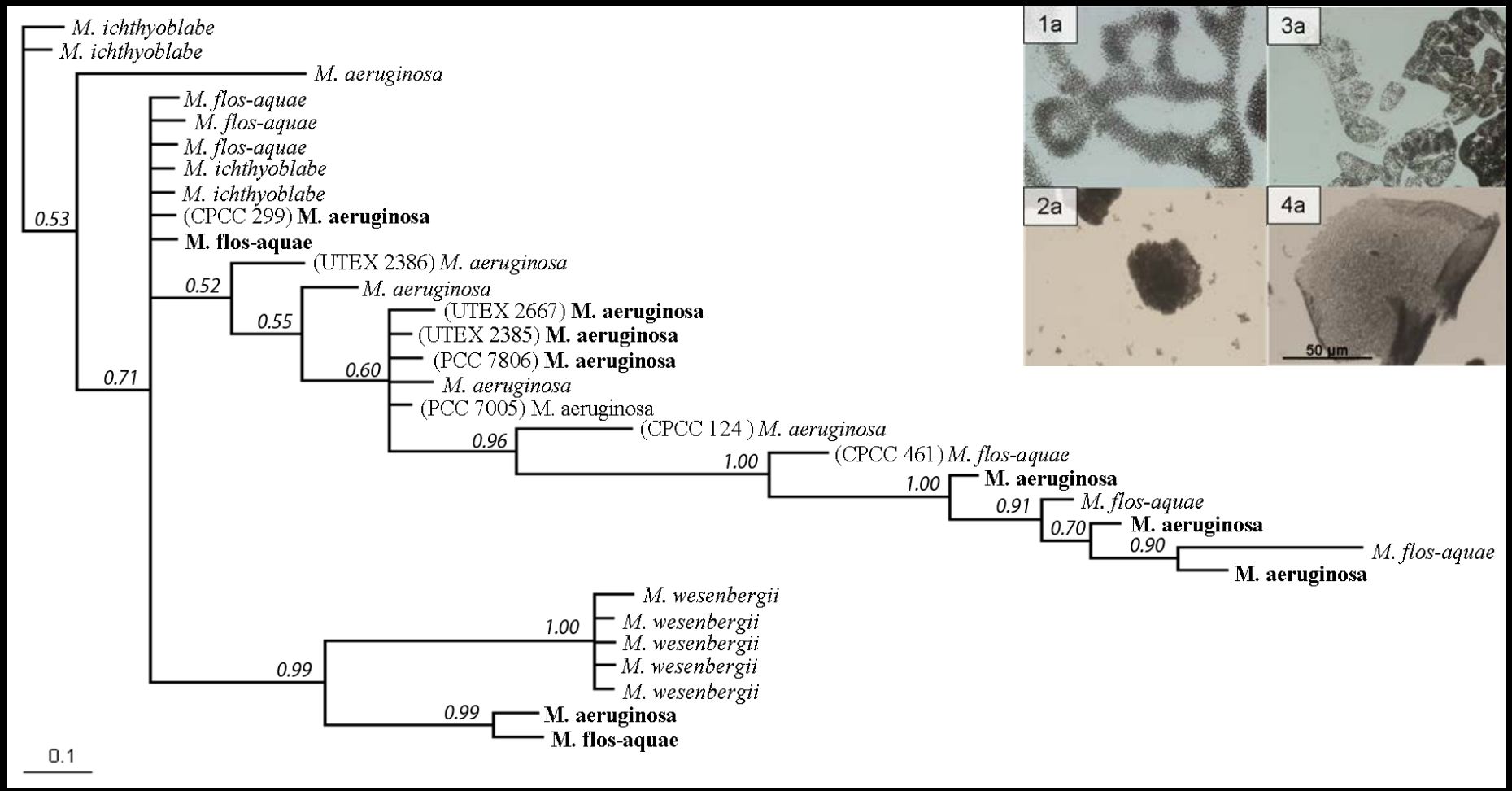
The Green Monster: China's Lake Taihu





Lake Taihu, contains at least 4 morphospecies of *Microcystis* sp.

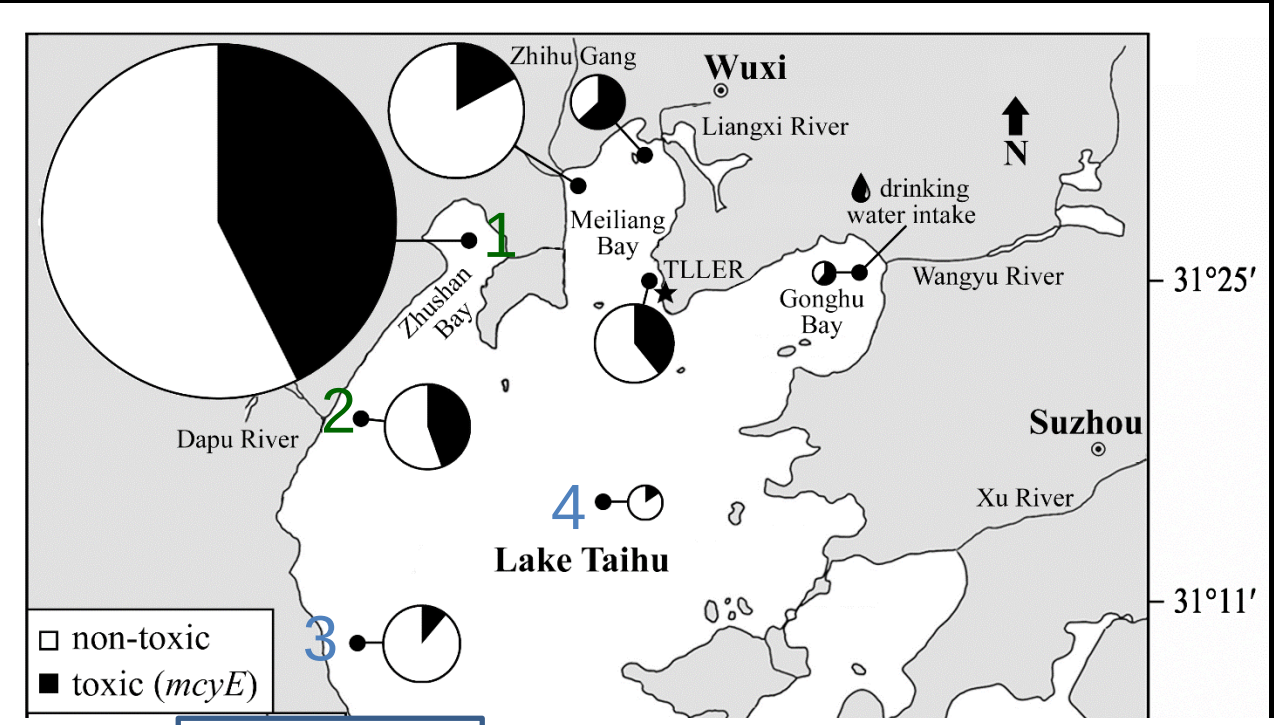
Most systems contain multiple, co-occurring species/strains/morphotypes



However, there is evidence for at least 8 different strains of *Microcystis* sp., half of which are able to produce microcystin

Otten and Paerl, 2011

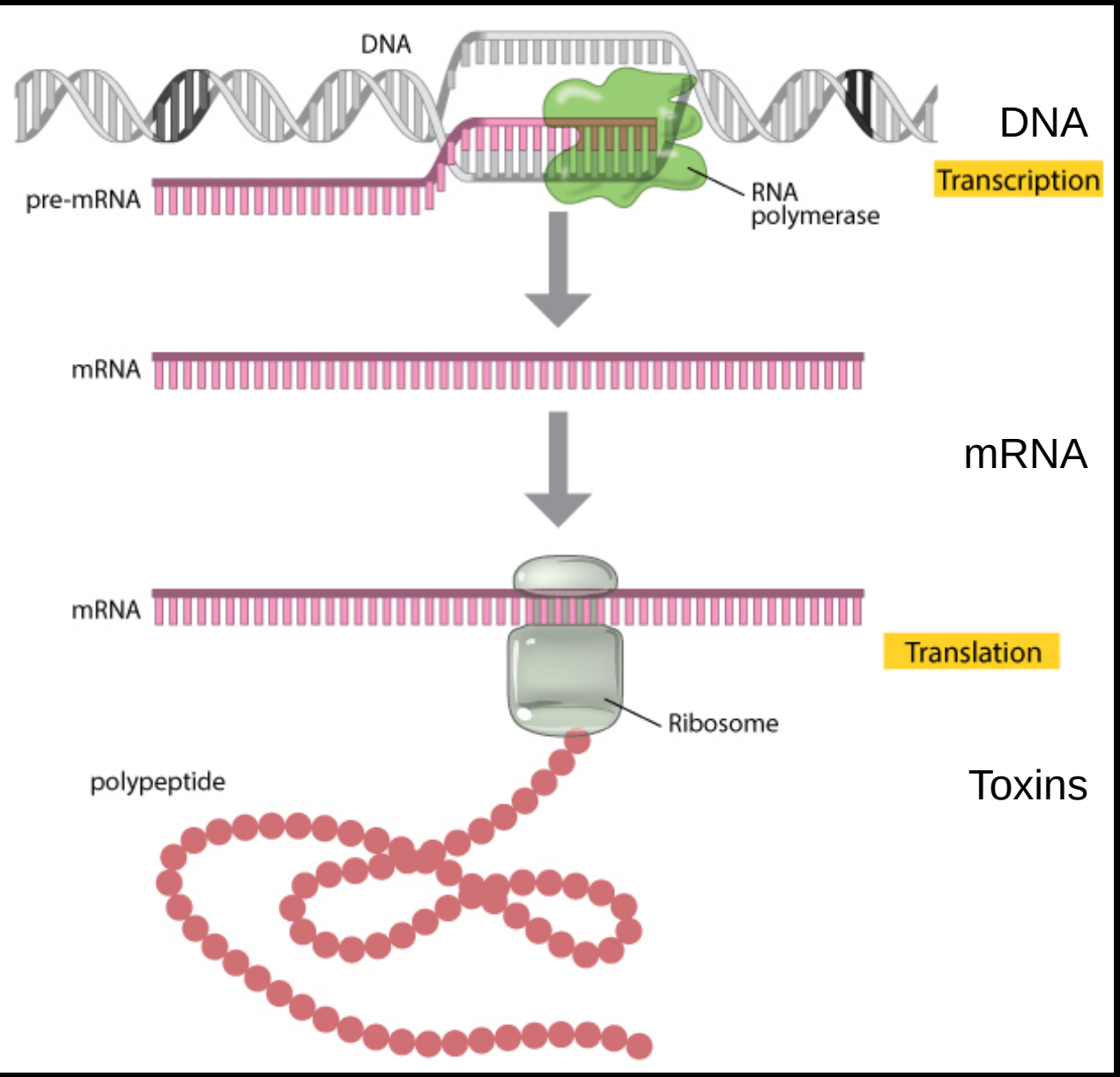
What conditions select for toxigenic strains over nontoxic ones?



sample site	temperature (°C)	ratio TN:TP	ratio DTN:DTP	avg. DTN (mg l^{-1})	avg. DTP (mg l^{-1})	chl *a* (μg l^{-1})	avg. MCs (μg l^{-1})	% toxic (*mcyE*)	turbidity (NTUs)
(1) Northwest (n = 12)	26.4 ± 3.1	17:1	29:1	3.62 ± 0.91	0.12 ± 0.10	192.1 ± 359.5	28.7 ± 6.6	42.6	26.4 ± 9.6
(2) West (n = 12)	26.8 ± 3.0	18:1	40:1	3.62 ± 0.86	0.09 ± 0.03	97.1 ± 92.3	8.2 ± 0.8	44.6	47.6 ± 23.3
(3) Southwest (n = 12)	25.7 ± 3.0	50:1	144:1	2.56 ± 1.00	0.02 ± 0.01	8.6 ± 8.9	1.1 ± 0.1	11.1	34.8 ± 18.0
(4) Lake Center (n = 12)	25.1 ± 3.1	58:1	132:1	2.64 ± 0.79	0.02 ± 0.01	7.1 ± 3.9	0.4 ± 0.1	15.5	23.9 ± 12.6
All Sites (n = 96)	26.3 ± 3.0	23:1	49:1	3.09 ± 1.06	0.06 ± 0.06	56.1 ± 147.9	6.4 ± 1.2	36.2	32.7 ± 20.3

[a]Sites (1) and (2) = high *Microcystis* biomass and toxigencity (*mcyE* possessing). Sites (3) and (4) = low *Microcystis* biomass and toxigenicity (*mcyE* possessing).

Pros and cons of targeting each level



DNA

Pro: can determine presence or abundance of toxigenic cells
Con: polyploidy and variable gene expression at different times complicate relationship with toxins

mRNA

Pro: gene expression should better reflect toxin production
Cons: mRNA is less stable than DNA, different genes within operon exhibit different expression patterns

Toxins

Pro: Toxins are ultimately what we are concerned about
Cons: very challenging to accurately quantify due to large numbers of toxin variants

*Confirmation of both toxic cells & toxins will provide best estimate of risk

Clancy & Brown, 2008

Pros & Cons of QPCR testing

- Pros
  - Faster than cell counting (2-3 hours from start to finish)
  - High throughput (40+ samples per analysis batch)
  - High sensitivity and specificity
  - DNA signal is amplified → good for early detection
  - Genes are better correlates of toxins than cell density
  - Cheaper than cell counting or toxin testing
  - Amenable to other targets (e.g, fecal bacteria)
  - Can be used for source-tracking or sample discovery

- Cons
  - Not a true substitute for toxin testing → tiered strategy
  - Cells must be intact to collect their DNA
  - Not useful on finished drinking water
  - Requires specialized equipment and training

Thanks for your attention!

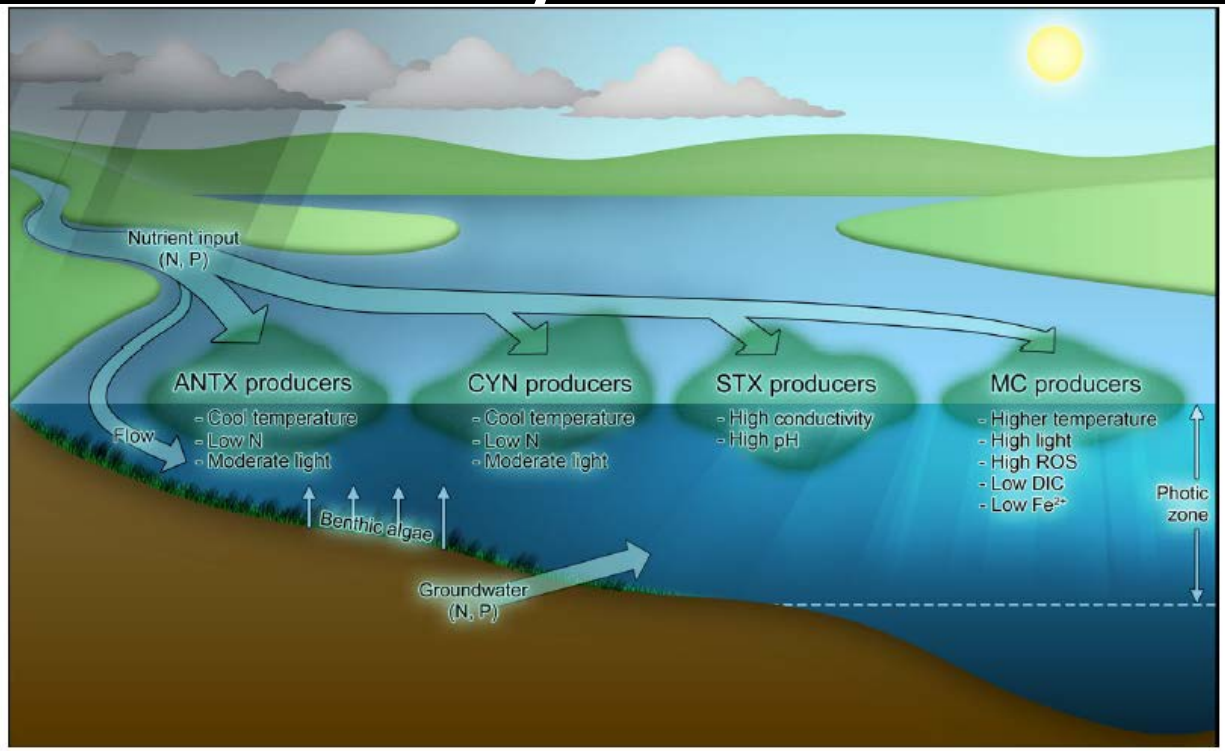


Fig. 1. Putative environmental drivers of cyanotoxin production that are presently recognized and the interplay of environmental stimuli on growth of toxigenic cyanobacteria.

Please feel free to contact me with any questions.

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