BELSPO

Molecular tools for diversity studies and toxicity detection

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1) **Diversity and taxonomy**

2) **Toxicity detection**

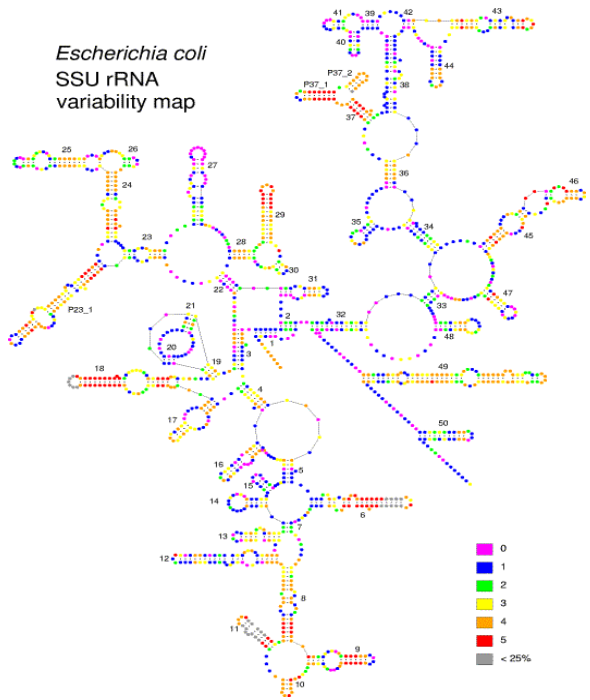
1) **Diversity and taxonomy**

2) **Toxicity detection**

Cyanobacteria are a group of photosynthetic bacteria of which the **taxonomy** has to face several problems:

1) Morphological simplicity
2) Morphological plasticity with environmental conditions

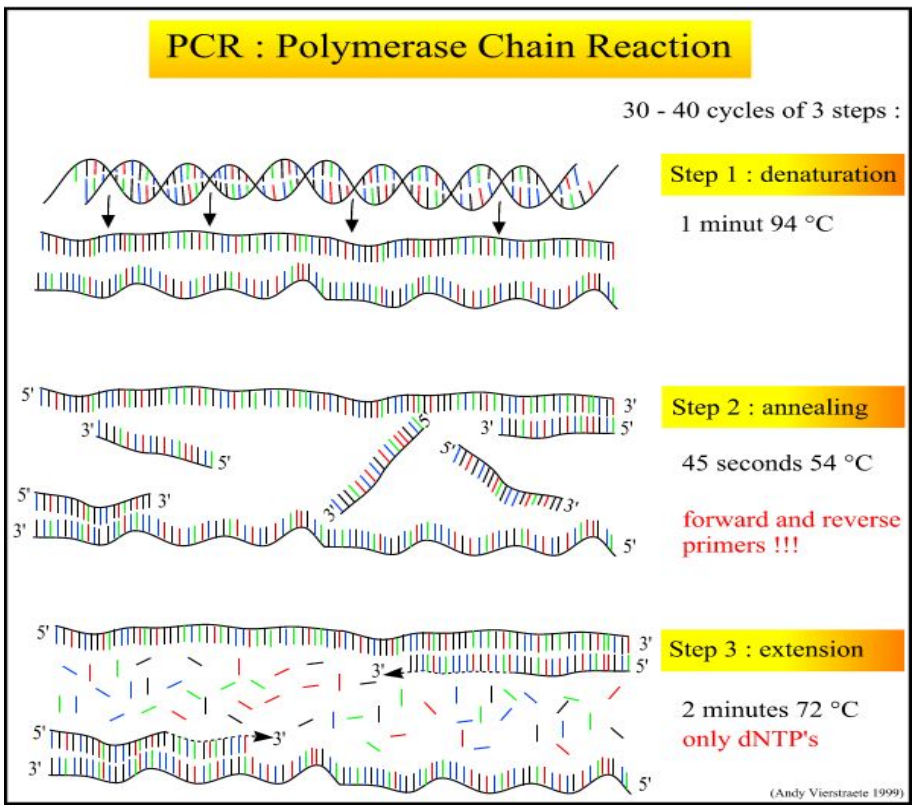
→ Use of **molecular** taxonomic markers to base the taxonomy on genetic information

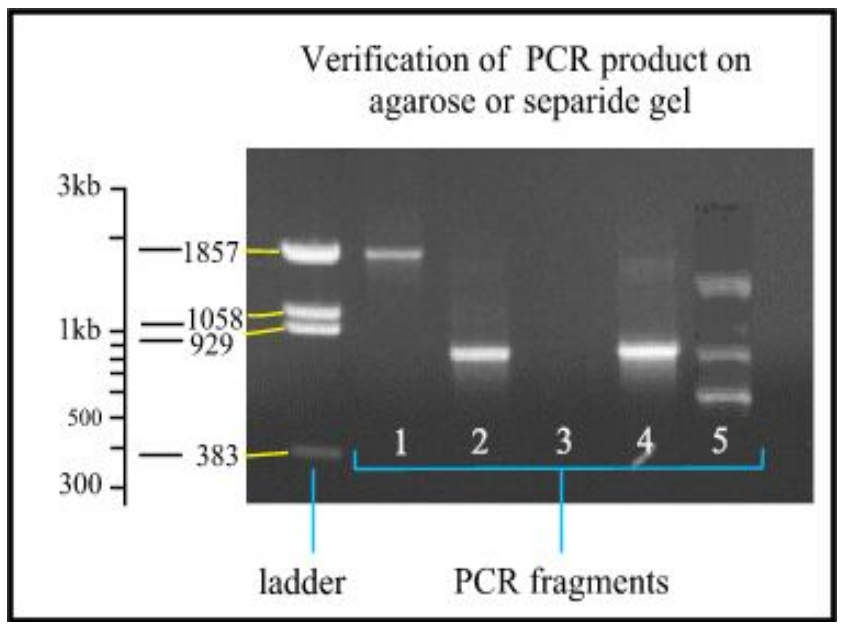


Classical marker for bacterial taxonomy : 16S ribosomal RNA

All molecular tools based on Polymerase Chain Reaction

1. Amplification of the gene of interest by PCR → millions of copies
2. Detection by electrophoresis on agarose gel → presence/absence of a band (PCR product) and/or sequence determination
3. Possibility of quantification of a PCR product





→ Sequencing

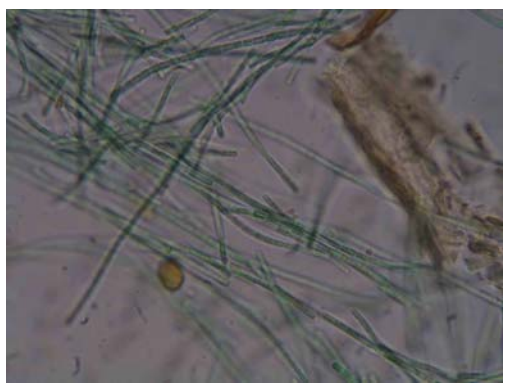
However, the morphological diversity does not generally coincide with genetic diversity :

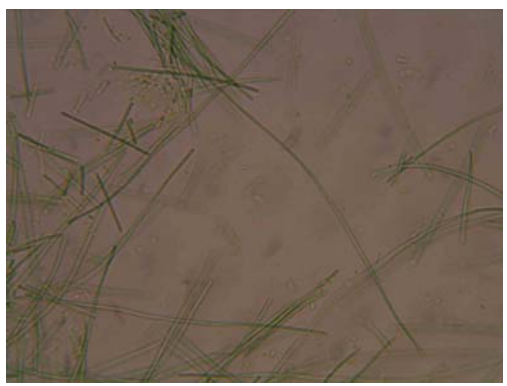
- a lot of different morphotypes corresponding to a conserved genetic marker

  ex: *Microcystis*

- a lot of genetic diversity hidden behind a simple morphology

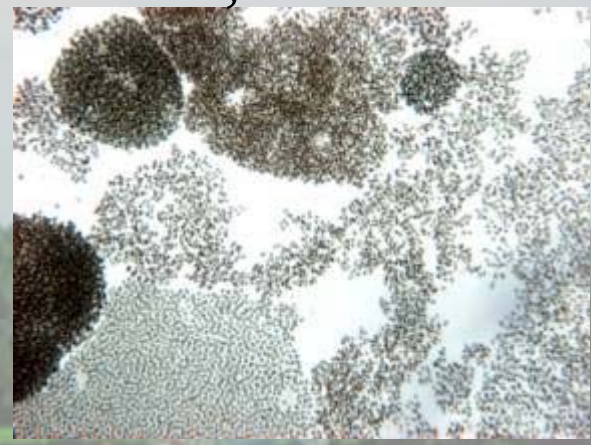
  ex: *Leptolyngbya*

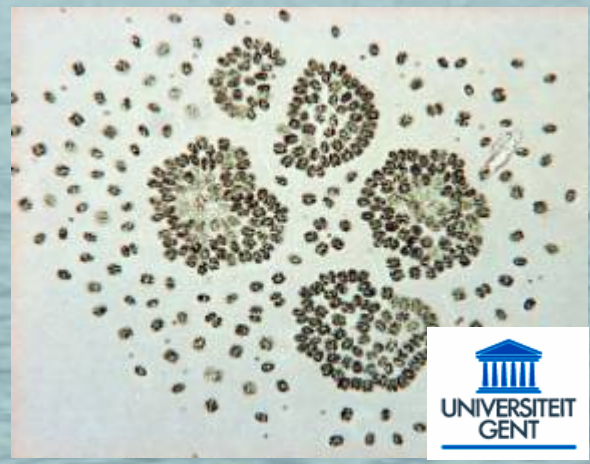




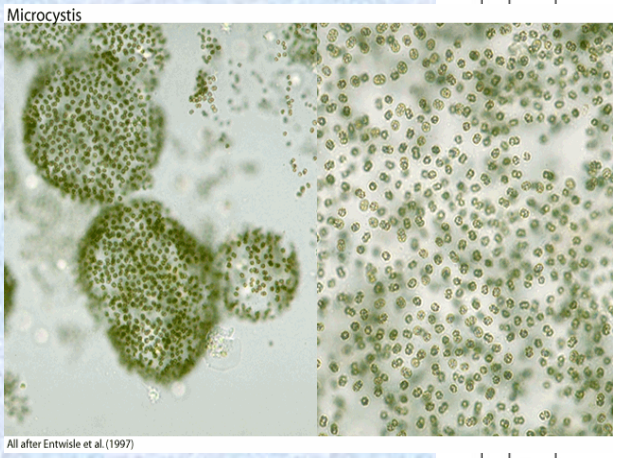
Microcystis

Unicellular, in colonies





(microcystines, anatoxine a et LPS)



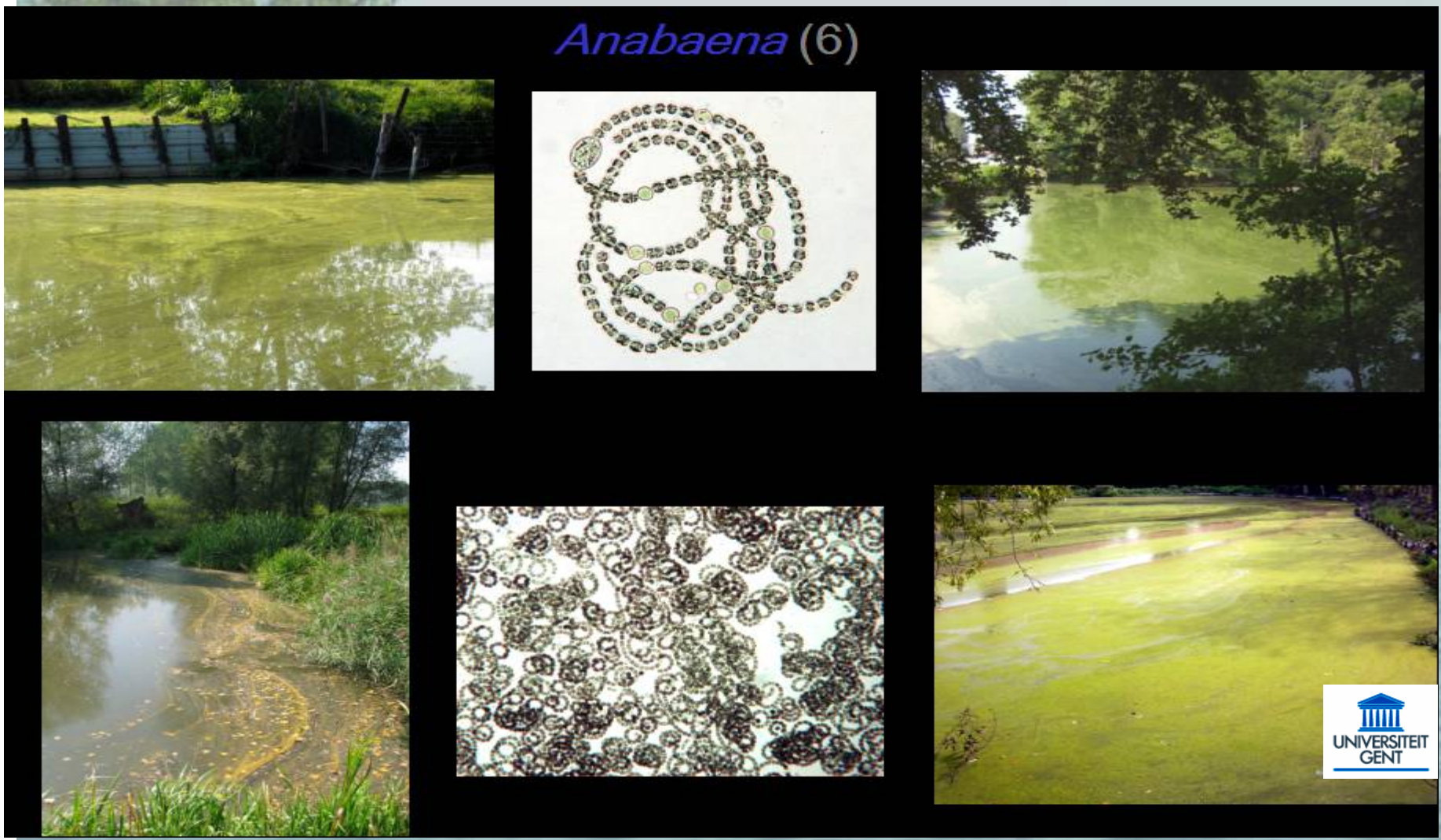
One tight 16S rRNA cluster but different morphologies

Otsuka at al. (2001): one species including *M. aeruginosa*, *M. ichtyoblabe*, *M. novacekii*, *M. viridis*, *M. wesenbergii*

Microcystis sp. (41)

- Microcystis sp. AWT139
- Microcystis aeruginosa PCC7820
- Microcystis aeruginosa NIES98
- Microcystis aeruginosa PCC7941
- Microcystis aeruginosa TAC86
- Microcystis ichthyoblabe TAC48
- Microcystis sp. P.R.China 4A3
- Microcystis sp. P.R.China 4B3
- Microcystis sp. KND9506
- Microcystis aeruginosa PCC7806
- Microcystis sp. 269
- Microcystis wesenbergii NIES111
- Microcystis wesenbergii NIES112
- Microcystis sp. NIES104
- Microcystis sp. 199
- Microcystis sp. PCC7941
- Microcystis sp. 130
- Microcystis sp. GL260735
- Microcystis sp. 205
- Microcystis wesenbergii TC7
- Microcystis aeruginosa TAC170
- Microcystis wesenbergii TAC52
- Microcystis sp. Thailand T17-1
- Microcystis sp. Thailand T1-4
- Microcystis aeruginosa NIES87
- Microcystis aeruginosa PCC7005
- Microcystis ichthyoblabe [AB012339]
- Microcystis novacekii TAC65
- Microcystis sp. GL280641
- Microcystis novacekii TAC20
- Microcystis viridis CC9
- Microcystis viridis NIES102
- Microcystis novacekii BC18
- Microcystis ichthyoblabe TC24
- Microcystis aeruginosa NC7
- Microcystis wesenbergii TAC38
- Microcystis aeruginosa TAC71
- Microcystis viridis TAC78
- Microcystis viridis TAC17
- Microcystis aeruginosa NIES89
- Microcystis wesenbergii NIES107

Filamentous, straight or coiled, with heterocysts (sometimes akinetes)



(Microcystines, Anatoxine a, Anatoxine a(s), LPS)

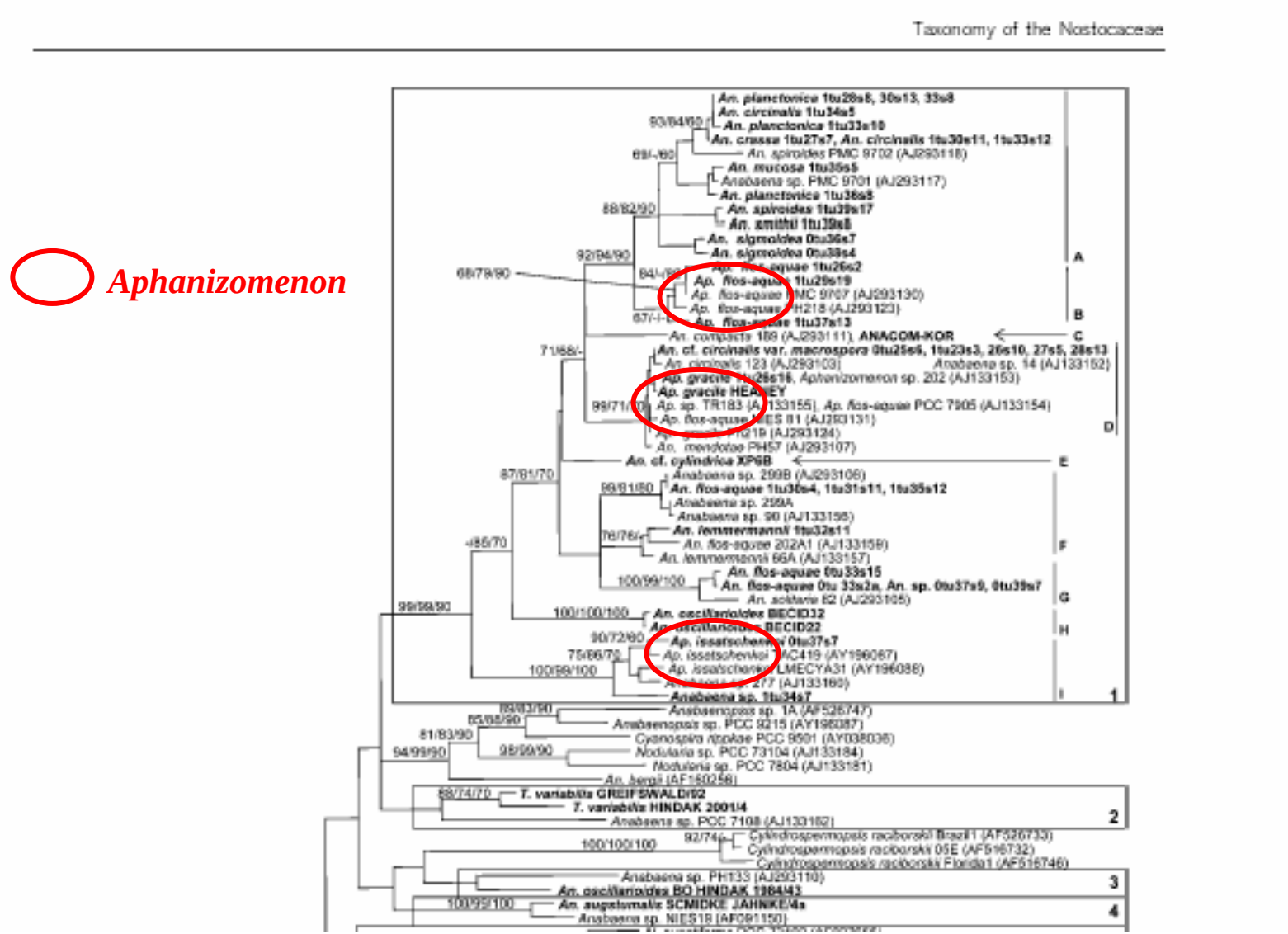
Aphanizomenon sp.

Filamentous, in bundles, with heterocysts (sometimes akinetes)

(microcystines + anatoxine a + saxitoxines + LPS)

CRITT BIO-INDUSTRIES
MIDI-PYRÉNÉES

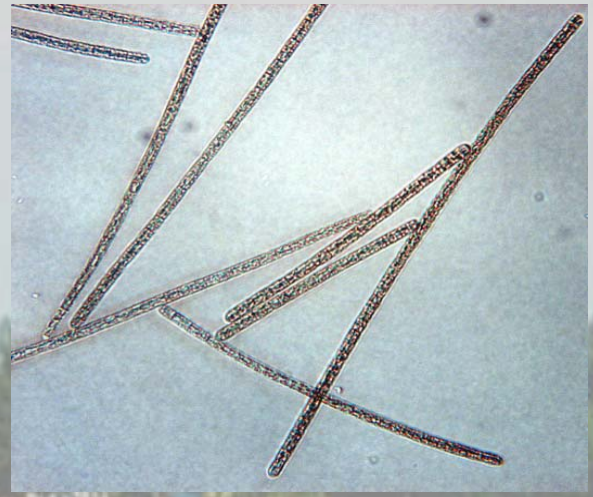
Very distinct morphologies but similar 16S rRNA sequences



Rajaniemi et al. 2005)

Planktothrix

Filamentous

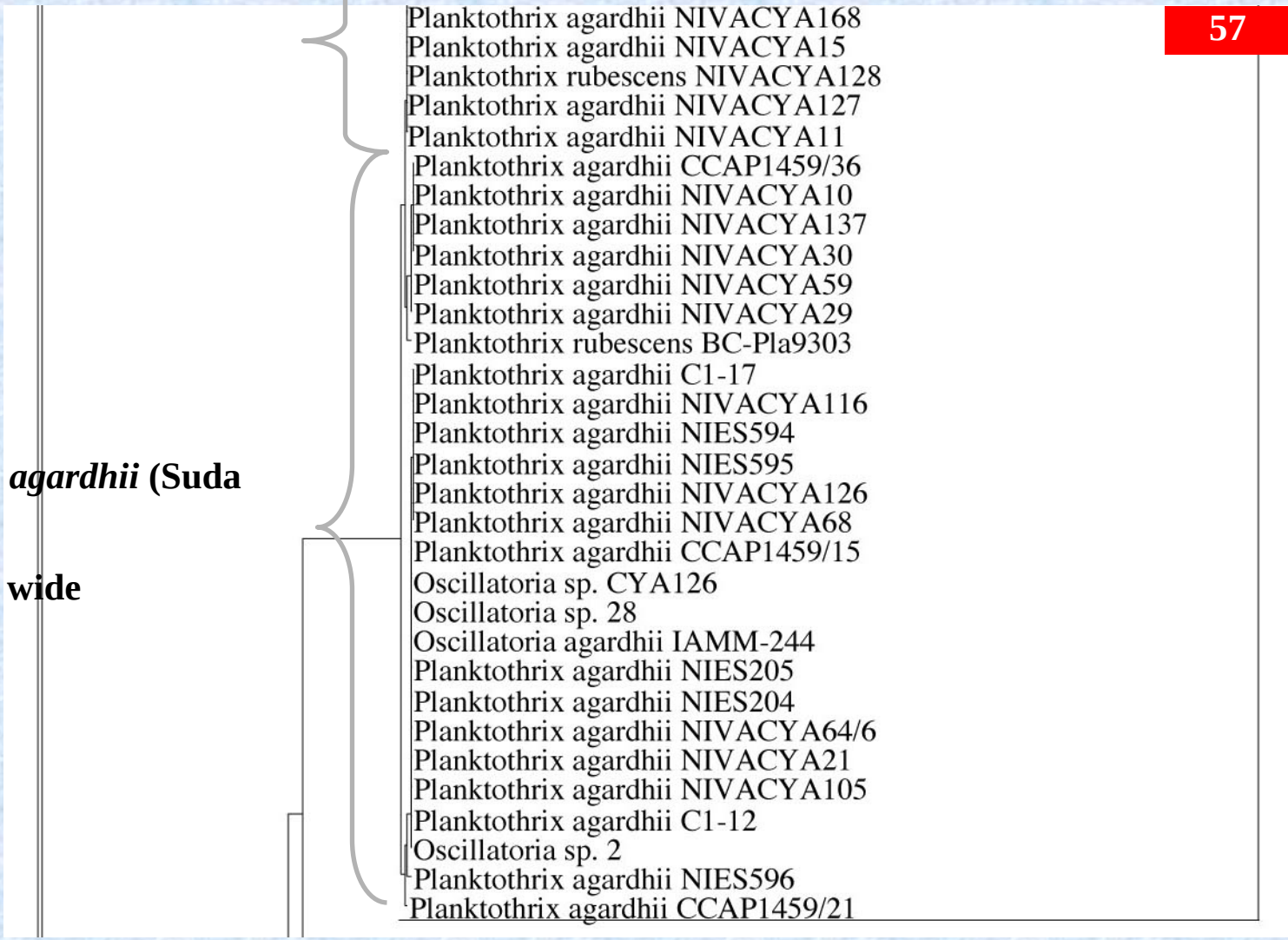


(microcystines, anatoxine a et LPS)

Good agreement between morphology and 16S rRNA sequences !

***Planktothrix agardhii* (Suda et al. 2002)**

2.3 – 9.8 µm wide



Usefulness

•Molecular diversity studies can tell us if the genotypes present in the sample belong to known toxin-producer, and thus can be used as an indication that further studies are needed.

•They also show the seasonal variations of bloom-forming taxa in one site.

•Presence/absence can be related to environmental parameters.

•Detection of invasions by exotic toxic cyanobacteria (*Cylindrospermopsis raciborski*, …)

Weakness

Toxic and non-toxic strains cannot be differentiated on the basis of 16S rRNA sequences (Lyra *et al* 2001)

except *Nodularia spumigena* of the Baltic Sea (Lehtimäki *et al* 2000)

1) **Diversity and taxonomy**

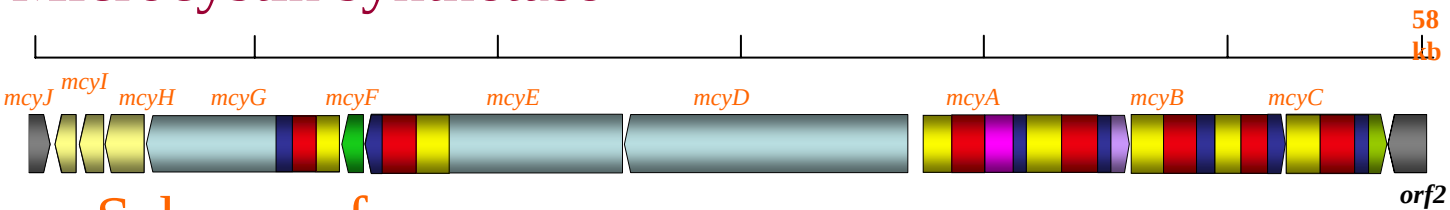
2) **Toxicity detection**

- Genes of toxin synthetase (microcystin)
- '*mcy* independent' genetic markers in *Microcystis* strains
- Working with single colonies
- DNA chips
- Real-Time Quantitative PCR

Molecular tools for toxicity detection

! Only the genetic potential to synthetize a toxin can be determined

1) Microcystin synthetase



Scheme of *mcy* operon

Shirai M. J. Biochim.,126 (1999): 520-529.

Microcystin synthetase is a long gene cluster (58 kb) and there are several genes that can be used for detection by PCR

2) Cylindrospermopsin synthetase

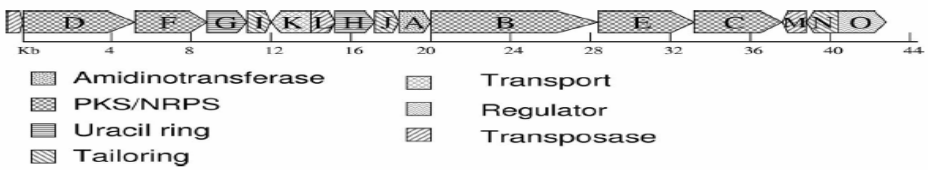
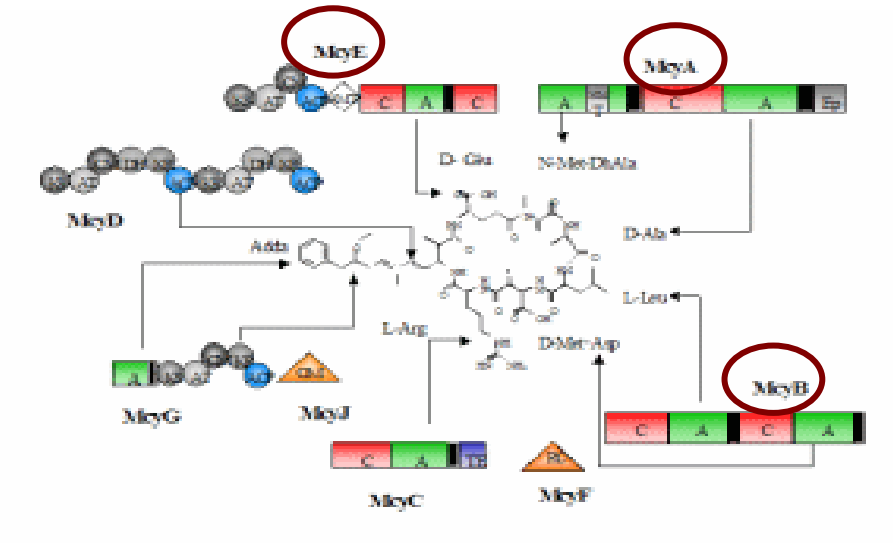


FIG. 2. Structural organization of the cylindrospermopsin gene cluster from *C. raciborskii* AWT205. Scale indicates gene cluster length in base pairs.

Cylindrospermopsin synthetase is a long gene cluster (42 kb) and there are several genes that can be used for detection by PCR

Mihali et al. 2008

mcyA, *mcyB* and *mcyE* are the most frequently used to detect strains of microcystin- producing *Microcystis* and *Planktothrix*

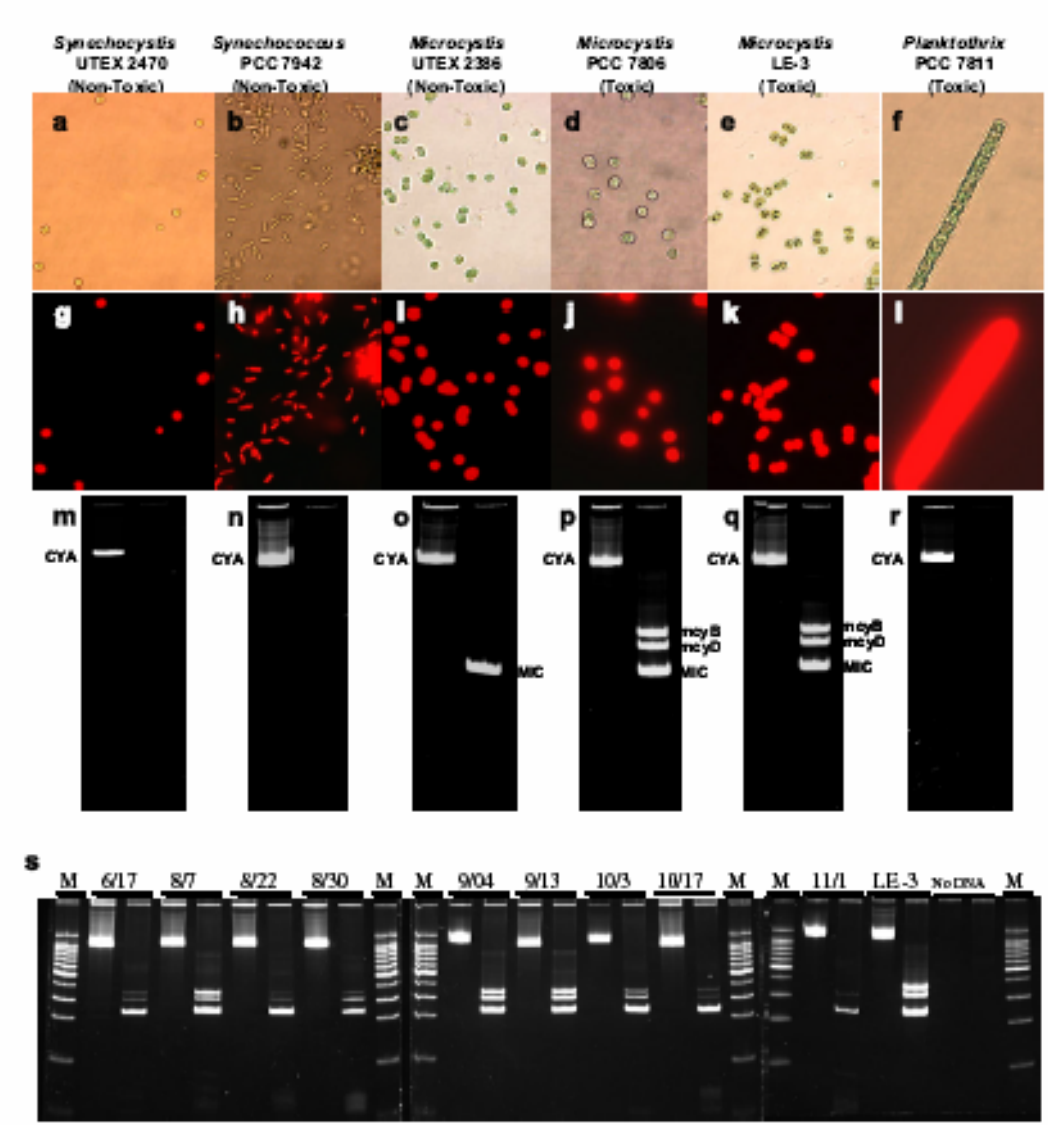


mcyE : synthesis of aminoacids Adda and Glu, that are important for toxicity of microcystins/nodularins, less variable than the other genes

→ *mcy*E gene is an adequate tool for the monitoring of cyanobacteria that are toxigenic (microcystin/nodularin)

http://www2.hu-berlin.de/biologie/moloeko/Research.html

Example of use of *mcyB* and *mcyD* for toxigenic *Microcystis*



Strains

Local pond

Ouellette & Wilhem, 2003

ANALYSES in B-BLOOMS1

STEP I: Microscopic observations: presence or absence of cyanobacteria

if cyanobacteria

STEP II: Detection of microcystin genes (*mcyB/mcyE*)

if *test* +

STEP III: Confirmation of microcystin presence by analytical methods

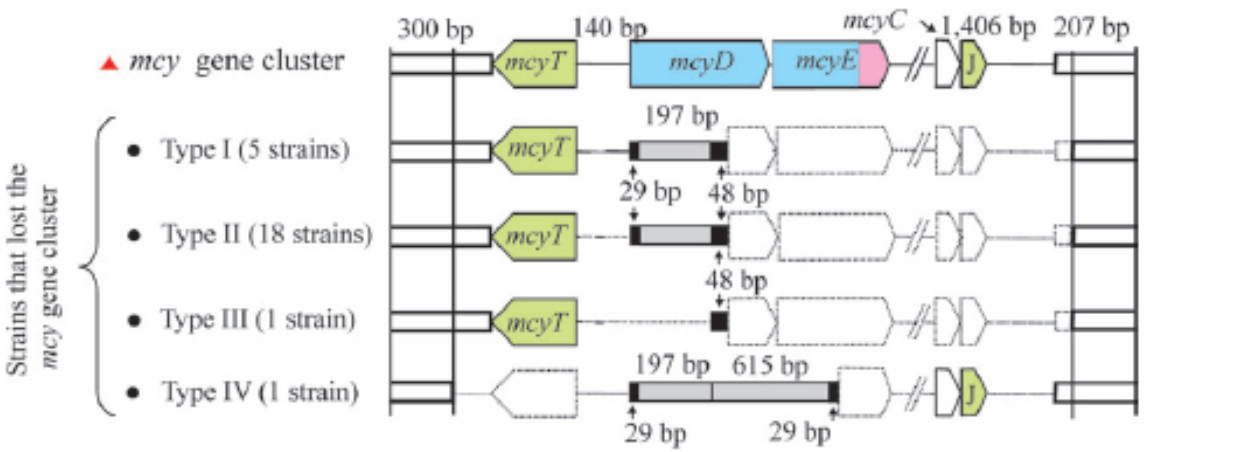
Of the 46 Flemish BLOOMNET samples positive for the two *mcy* genes, 54% actually contained total microcystins, in a range of concentration from 18 to 2651 µg [g DW]$^{-1}$

OK:

Constitutive transcription of *mcy* genes at a low level, increased with light or other factors (Kaebernick et al. 2002)

KO:

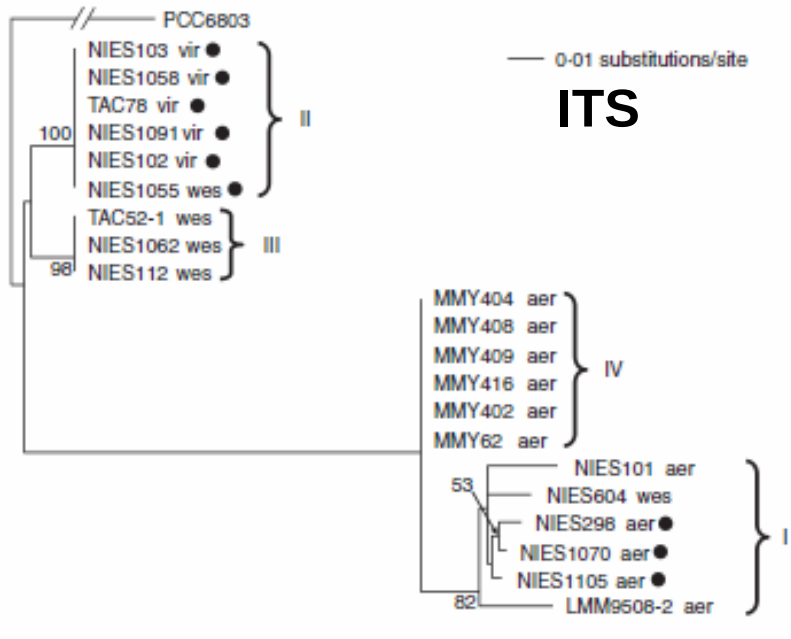
Gene deletions can produce non-toxic strains (Christiansen et al. 2007)

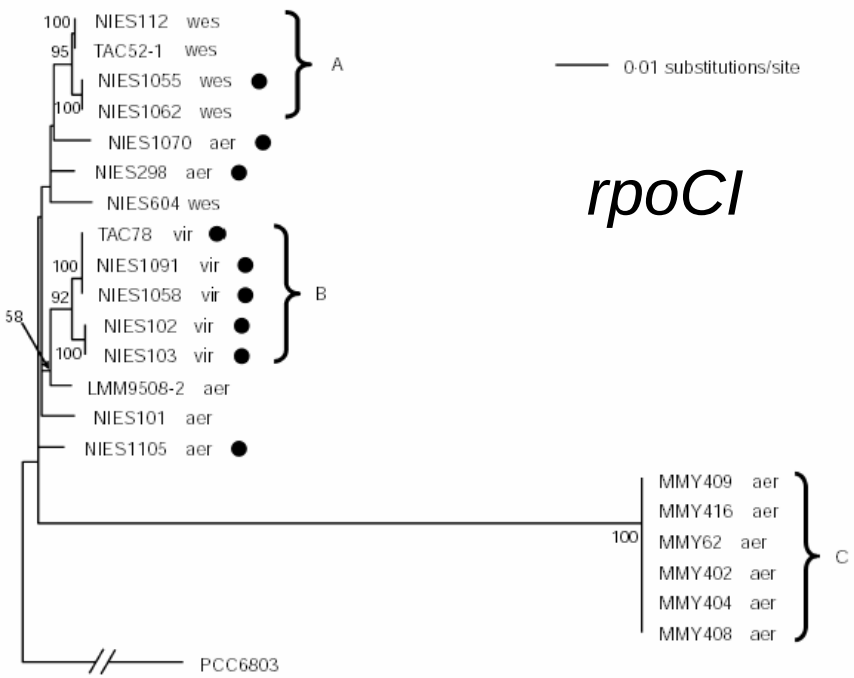


→ False positive answers are possible

mcy genes not always adequate → other molecular tools

'*mcy* independent' genetic markers in *Microcystis strains*





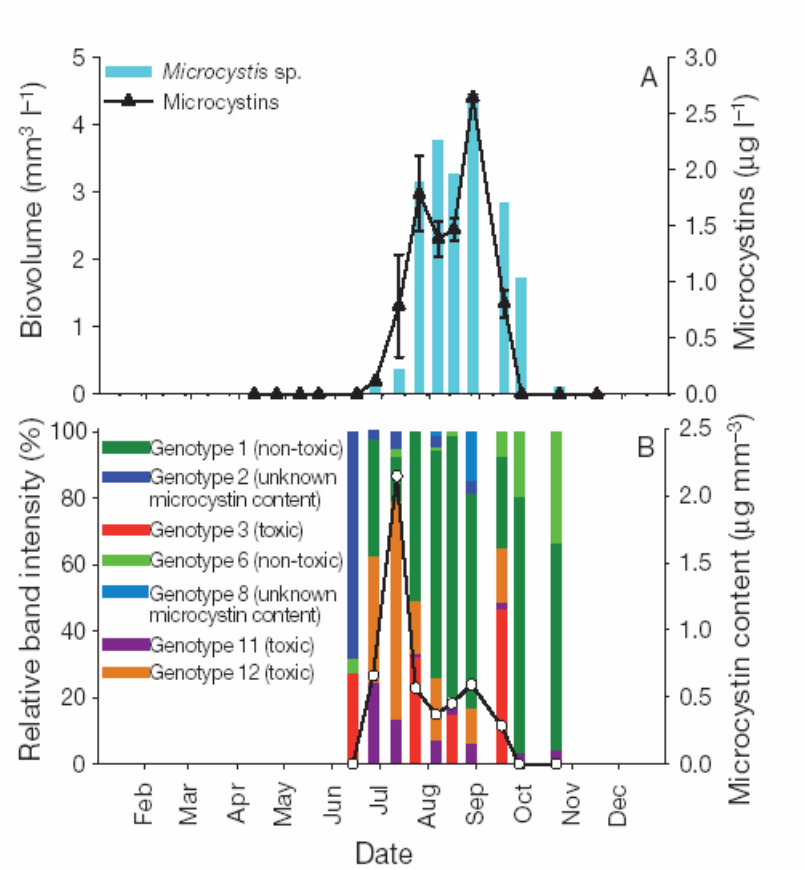
- Toxic strains

Yoshida et al. 2008

Working with single colonies instead of blooms samples

Colonies in one bloom sample can be toxic or non-toxic

Janse et al. (2004) finds a relation between toxicity and the sequence of ITS (spacer in the rRNA operon)

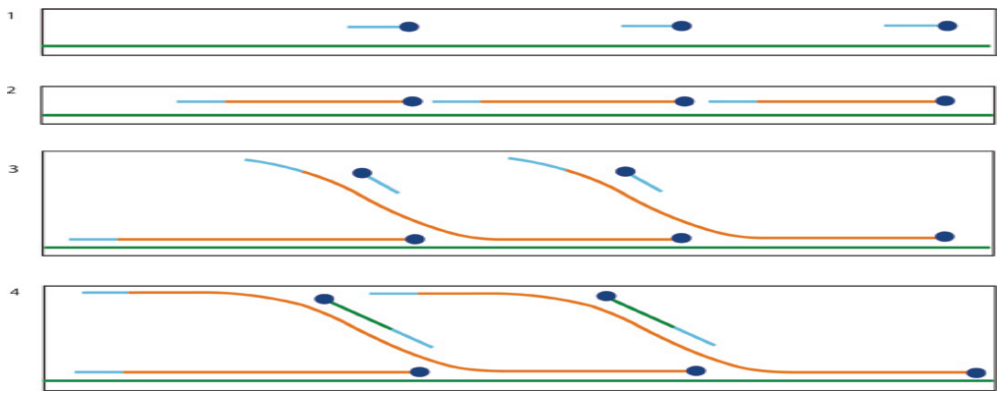


Seasonal dynamics in a Dutch lake : more non-toxic genotypes at the end of the bloom

Working with single colonies instead of blooms samples

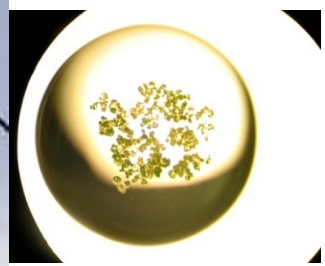
Importance of studying single clones/filaments as the relative proportion of toxic/non-toxic genotypes will determine the quantity of microcystin

New method (Whole Genome Amplification) to amplify whole genome up to 5 billion-fold from a single cell (Raghunathan et *al.*, 2005)



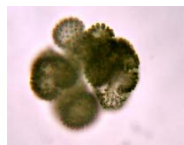
This allows multiple PCR reactions with different genes and solves the problem of ‘unculturable’ taxa

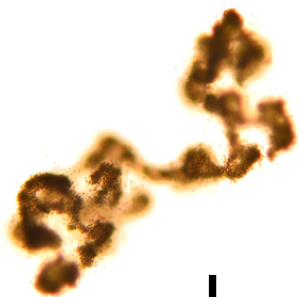
Sample reception



Isolation under stereoscopic microscope

Picture and control under microscope





Storage in PCR tubes in a small known volume

-Whole genome amplification

-PCR with cyanobacterial specific primer

-Sequencing

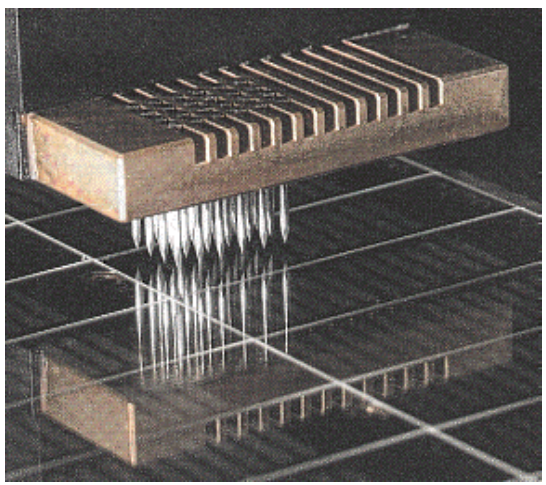
-Analysis

Conclusions

Working with single colonies/filaments seems merely useful for research purposes. It should help to elucidate the population genetic make-up and dynamics.

An emergent technology: DNA chips

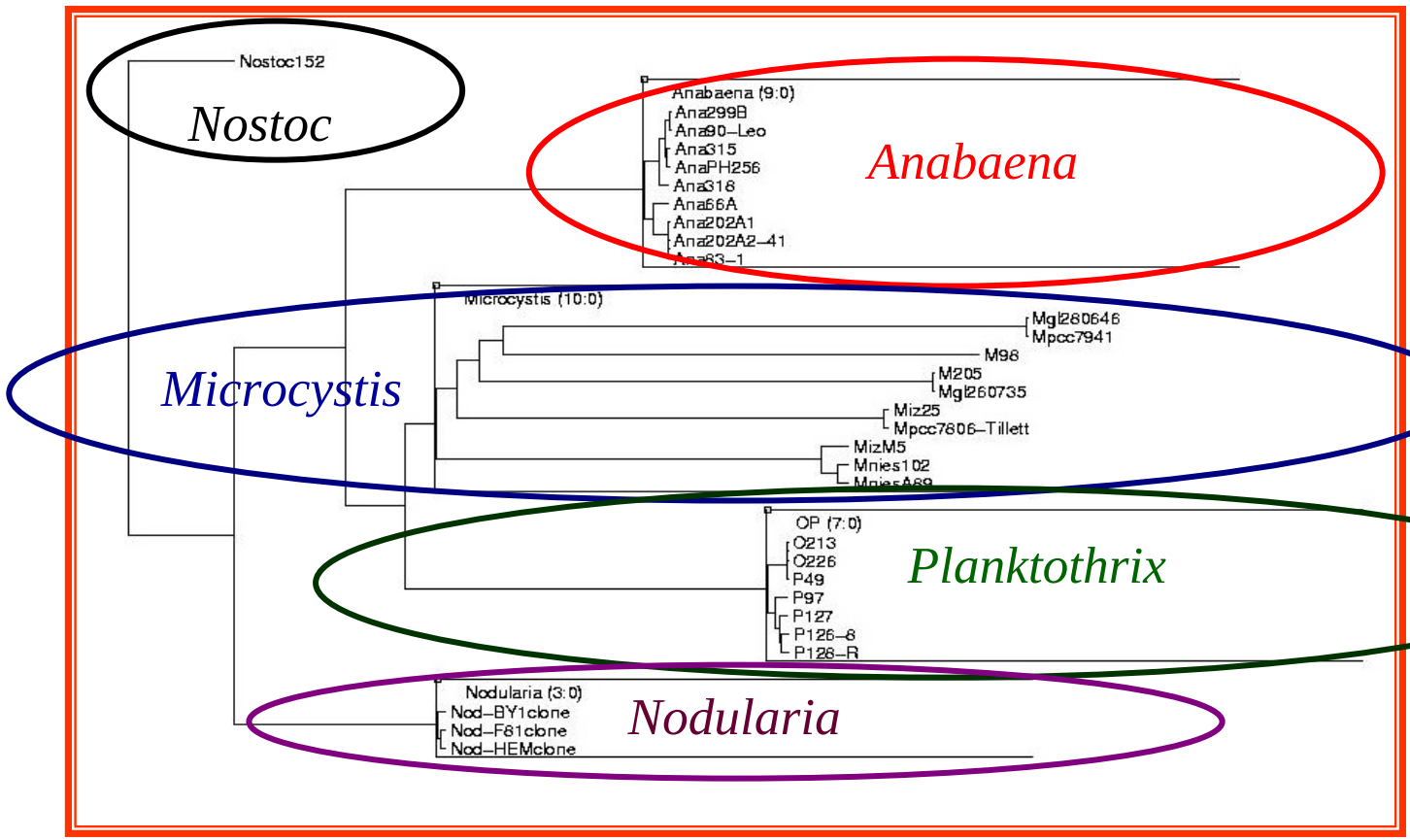
On a support (glass slide) are deposited and linked thousands (or more) tiny spots of a few µls containing probes (DNA fragments) that are able to recognize and hybridize with a specific sequence of DNA of a sample. Hybridization is detected because the sequence that will hybridize is labelled with fluorescence.



Frouin & Gidrol (2005) Biofutur 252

A high-throughput method that can be automatized

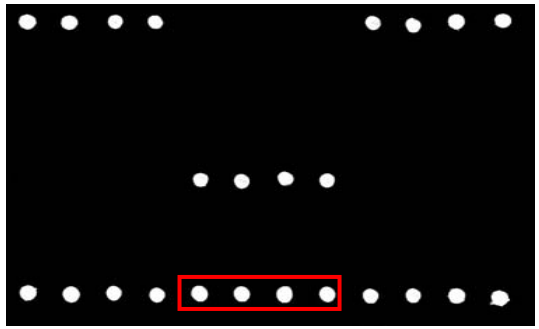
Toxic Cyanobacteria - *mcy*E gene



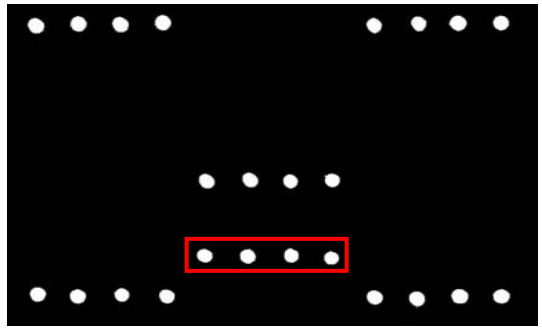
→possible to design **genus-specific** probes/primers for PCR

(slide by Anne Rantala)

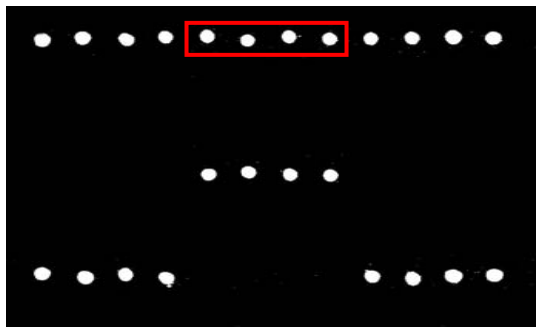
Some results on strains



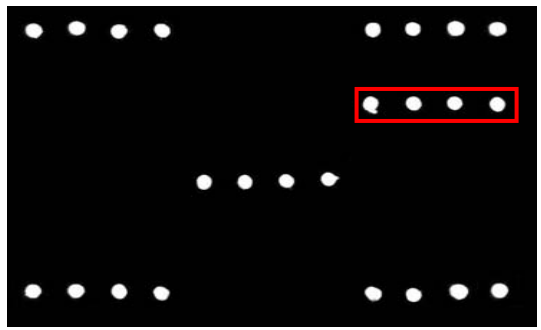
Aphanizomenon sp. 202
Lake Vesijärvi, Finland



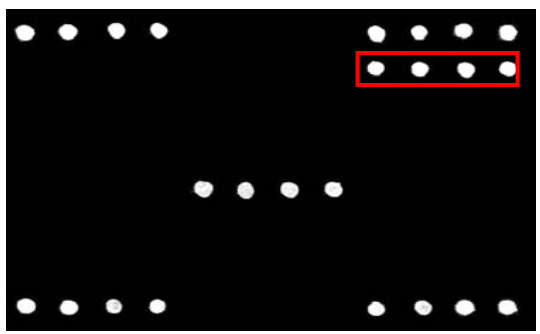
Calothrix sp. PCC7714,
Pool, India



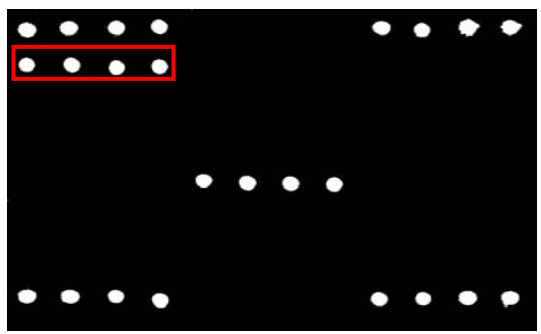
Microcystis wesenbergii
NIES104, Freshwater, Japan



Planktothrix 1LT27S08
Lago Trasimeno, Italy



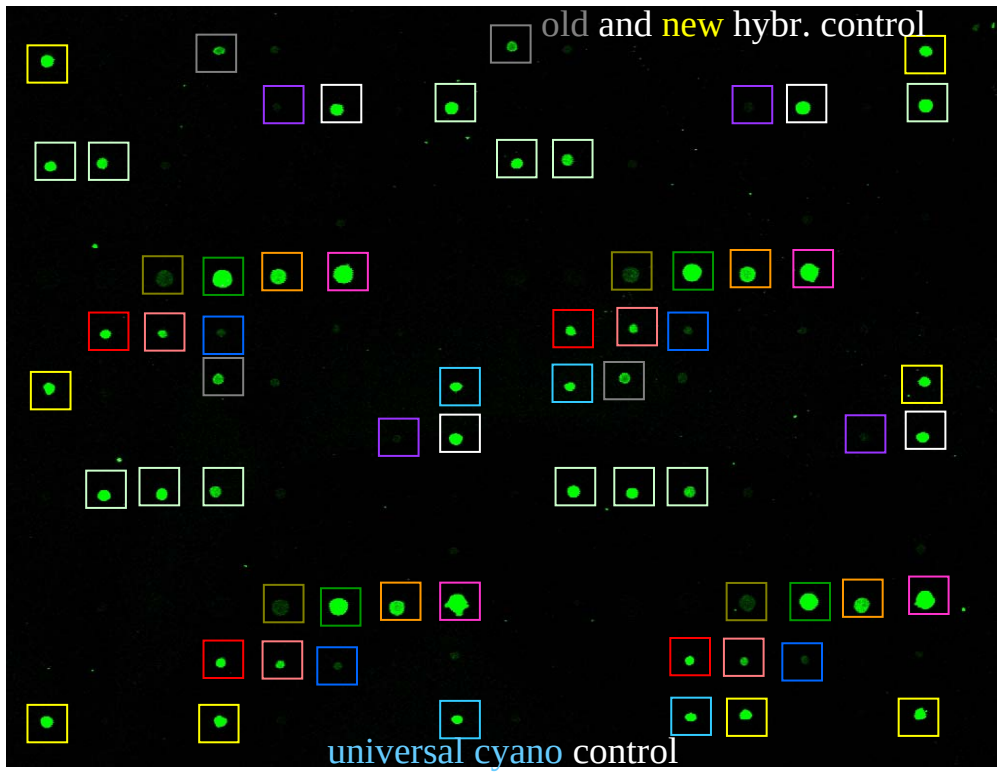
Spirulina subsalsa PCC6313



Synechococcus Heg 74-30,
Lake Kuusjärvi, Finland

Environmental sample from Lake Tuusulanjärvi

- Signals from 16S rRNA spots
  - *Microcystis*
  - *Synechococcus*
  - *Anabaena/Aphanizomenon*
  - *Leptolyngbya*
  - *Woronichinia*
  - various *Anabaena/ Aphanizomenon* subgroups e.g. toxic *Anabaena*
  - *Snowella*
- With other methods (microscopy, strain isolation, cloning, DGGE) the same groups were detected in Lake Tuusulanjärvi samples



Signals from *mcyE*-spots

- *Anabaena-mcyE* and *Microcystis-mcyE*

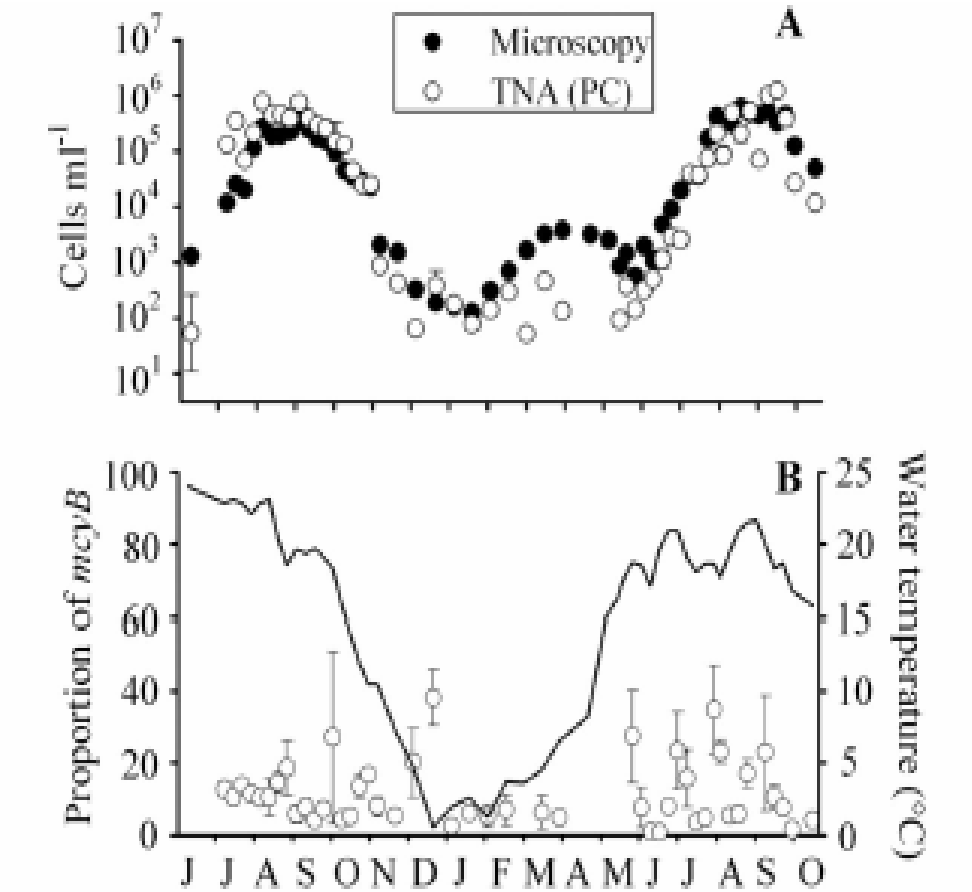
(slide by Anne Rantala)

Conclusions

- DNA chips can be used as monitoring tools (and for research)
- Well designed probes can give more information than microscopy (e.g. presence of *mcyE* genes) in one operation
- Thanks to the multiplicity of the probes, the DNA chips can give a global view of the diversity (in contrast to PCR reactions targeting one or few genes)
- Flexible design to integrate new toxin genes when the sequences will be determined (neurotoxins)

BUT need of a special non-routine equipment

Quantification by Quantitative Real-Time PCR



Agreement between counts of *Microcystis* by microscopy and numbers of gene copies

Proportion of *mcyB* genotypes in the total *Microcystis* population and relation to water temperature

Kurmayer & Kutzenberger 2003

Quantification by Quantitative Real-Time PCR

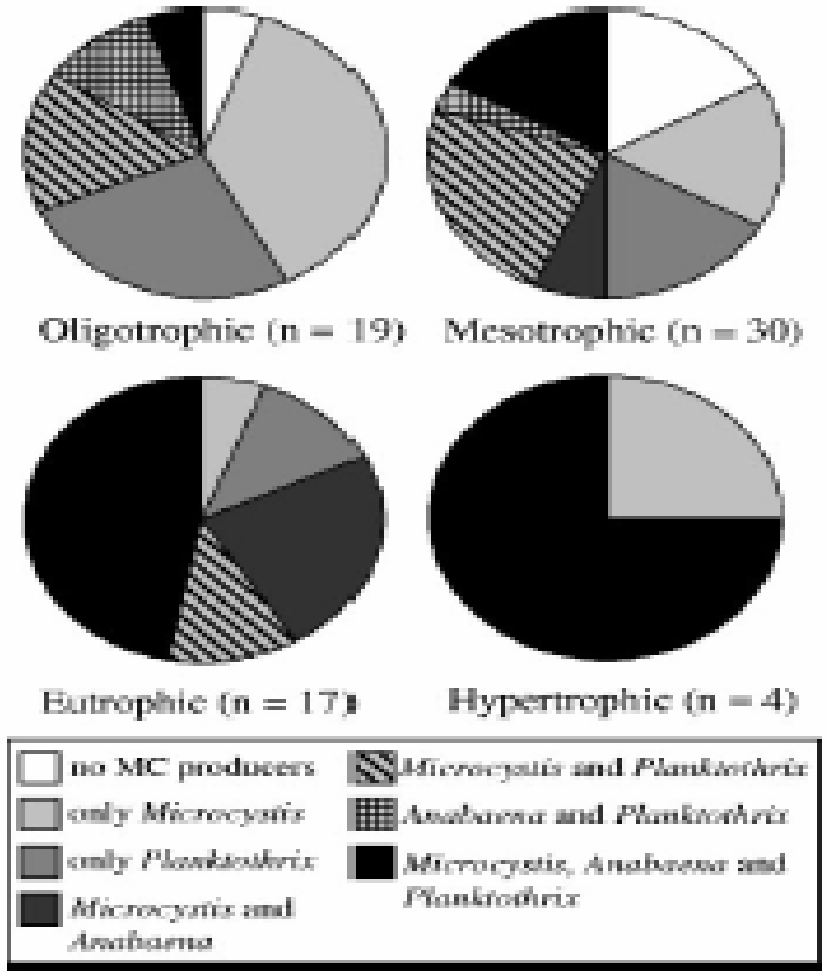


FIG. 2. Proportion of lakes with different combinations of potential MC producers, *Anabaena*, *Microcystis*, and *Planktothrix*, based on the presence of genus-specific *mcyE* genes in oligotrophic (TP, <10 μg/liter), mesotrophic (TP, 10 to 34 μg/liter), eutrophic (TP, 35 to 100 μg/liter), and hypertrophic (TP, >100 μg/liter) lakes.

mcyE of *Microcystis*, *Planktothrix* and *Anabaena* in lakes of different trophic status

Rantala et al. 2008

Conclusions

With technological improvements, and well designed probes, the quantitative Real Time PCR could be useful to monitor bloom developments at the diversity and toxigenicity levels and quantify the copy numbers of toxin genes in the samples.

VEOLIA (France) is busy to develop such an automated quantitative PCR system.

Final conclusions

• Only molecular techniques can tell which of the cyanobacterial genera present in a sample is the actual toxin producer

• Molecular techniques are very sensitive compared to most chemical methods

(Dittmann & Börner, 2005)

→ Potential tool for **early-warning** systems