

**A manual for the use of in situ genetic techniques
to quantify genotypes of cyanobacteria in freshwater under non-bloom
conditions and to predict cyanopeptide occurrence under bloom conditions**

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1) Introduction

Cyanobacterial toxins are amongst the most ubiquitously found potentially hazardous substances in surface waters used by humans. Though these substances are natural toxins, eutrophication (i.e. excessive loading with fertilising nutrients) has caused massive cyanobacterial proliferation throughout Europe. Thus, cyanotoxins now occur with unnatural frequency and concentration. In some cases this even applies to deep-layer species in less eutrophic reservoirs.

A large group among the diverse cyanobacterial toxins are the oligopeptides (termed “cyanopeptides” in the following). The strongly hepatotoxic and tumour-promoting microcystins were the first oligopeptides to be intensively studied. Recently, substantial progress in elucidation of the structures and the biosynthesis of other cyanopeptides has been made, and preliminary information on toxicity of microviridins, aeruginosins, cyanopeptolins, microginins and other cyanopeptides is emerging (Dittmann et al. 2001). Many cyanopeptides are protease inhibitors, i.e. inhibiting trypsin/chemotrypsin [aeruginosin (Kodani et al. 1998), cyanopeptolin (Jakobi et al. 1995)] or serine (microviridins D-F, Shin et al. 1996). Pharmacological research is increasingly detecting bioactive, thus potentially toxic, substances in cyanobacteria (Moore et al. 1996). Toxicity testing of crude extracts has demonstrated toxicity beyond that predicted from microcystins (Jungmann & Benndorf 1994, Keil et al. 2002). Recently, oligopeptides (oscillapeptin, microviridin J) with a toxicity to aquatic crustaceans that is comparable to that of microcystin have been reported (Agrawal et al. 2001, Blom et al. 2003, Rohrlack et al. 2003).

Cyanopeptides are small peptides comprising a few amino acids and are produced by several genera of planktonic freshwater cyanobacteria, e.g. *Anabaena*, *Microcystis* and *Planktothrix* (see Fig. 1) (Moore et al. 1996). Most research has been performed on the elucidation of biosynthesis of microcystins showing that the microcystins are members of a peptide family which have the common structure cyclo (D-Ala-L-X-D-MeAsp-L-Z-Adda-D-Glu-Mdha), where X and Z are variable L-amino acids, Adda is 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl-4,6-decadienoic acid, D-MeAsp is 3-methyl-aspartic acid and Mdha is N-methyldehydroalanine (Carmichael et al. 1988). More than 70 structural variants of microcystins are known to date. Microcystins are synthesised by the thiotemplate mechanism like other non-ribosomal peptides (i.e. antibiotics such as gramicidin or tyrocidin) produced by bacteria and fungi (Marahiel et al. 1997). The large enzyme complex encoded by the *mcy*

gene cluster is composed of peptide synthetases, polyketide synthases and tailoring functions for microcystin biosynthesis. It has a modular structure, each module activating and incorporating specific constituents of the heptapeptide (Tillett et al. 2000). The *mcyA*, *mcyB* and *mcyC* genes are responsible for the activation and incorporation of Mdha, D-Ala, L-X, D-MeAsp, L-Z of microcystins during biosynthesis.

In the laboratory it has been shown that one species usually comprise a range of different “chemotypes”, i.e. morphologically indistinguishable individuals containing different peptides (Fujii et al. 2000, Fastner et al. 2001). It has further been shown that this diversity does exist in the field, for example microcystin-producing vs. non-microcystin producing strains have frequently been isolated from the same water sample (Ohtake et al. 1989, Vezie et al. 1998, Rohrlack et al. 2001). In addition a high diversity of cyanopeptides such as microcystins, anabaenopeptins, aeruginosins, cyanopeptolins has been demonstrated in individually selected colonies of *Microcystis* in Lake Wannsee (Fastner et al. 2001).

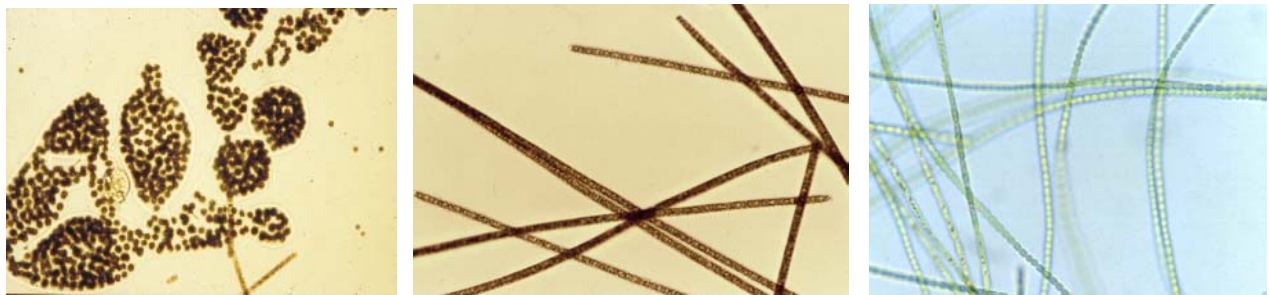


Fig. 1: Cyanobacteria from surface water potentially producing cyanopeptide hazardous substances: *Microcystis* (left, 400×), *Planktothrix* (middle, 400×), *Anabaena* (right, 200×)

Based on this diversity observed in the laboratory and in the field the waxing and waning of microcystin-producing vs. non-microcystin-producing strains has been suggested as a most important factor regulating microcystin net production in water (Sivonen & Jones 1999). However, the quantification of toxic genotypes versus non-toxic genotypes was long time impeded because these genotypes cannot be differentiated in the microscope. Therefore the factors leading to cyanobacterial blooms consisting of either microcystin-producing or non-microcystin producing species have not been identified. This lack of knowledge is especially relevant to the even less studied cyanopeptides as a group. In general factors studied to influence microcystin production included nitrogen, phosphorus, temperature, light, micronutrients (iron, molybdenum), pH and alkalinity etc. (Sivonen & Jones 1999). Laboratory studies suggest that toxin production is coupled to cyanobacterial biomass (Orr & Jones 1998). The situation becomes more difficult in the field since several different

genotypes of one species can coexist and therefore influence the toxin concentration in the biomass and water. In nature aquatic cyanobacteria not only vary in abundance by three to four orders of magnitude over the year but large variations in microcystin contents between sites from non detectable to up to 0.2 - 0.3% of dry weight have been observed (Fastner et al. 1998) as well. It might be speculated that sites and waterbodies differ in genotype composition by a factor of 10 or more. The important aim in the near future will be to identify the factors that govern genotype and chemotype composition in natural populations.

This manual aims (i) to describe recently developed techniques for the use of *in situ* quantification of genotypes producing specific cyanopeptides and (ii) to provide information on standard methods in water analysis useful to characterize environmental factors associated with the occurrence of cyanobacteria in general and to relate the stability of genotype composition throughout the year. It is beyond the scope of this manual to describe each standard method in water analysis in detail and it will be referred to standard literature instead. Information on environmental factors in relation to genotype composition is essential to predict cyanopeptide occurrence under bloom conditions in the near future. Because so far most research has been performed on the toxic microcystins all techniques currently available include the quantification of microcystin-producing genotypes in *Microcystis* sp. We believe that all techniques would work equally well for other cyanopeptide genotypes.

2) Lake sampling

The following sampling protocol has been developed among partners of WP2 and has been used in field work during 2003 and 2004. It is the aim of the net sample to provide a comparable but more concentrated picture of the algae in the lake, especially in mesotrophic and oligotrophic lakes. In eutrophic lakes the net sample is less important. A net sample is taken by vertical net hauls (30 µm mesh size) over the total water column in shallow lakes and over the euphotic zone in deeper lakes, e.g. Lake Wannsee is shallow and polymictic (max. depth 8m) and the total watercolumn will be sampled; in Lake Mondsee the euphotic zone is ca 15 m, consequently a net haul from 20 m will be sufficient. Try to obtain several 100 ml of the sample showing at least some green colour. If necessary repeat the vertical net haul until colour of the sample is achieved.

A quantitative integrated sample is taken by mixing samples from various depths (e.g. 0 m, 3 m, 6 m, 9 m, 12 m, 15 m, 20 m in Lake Mondsee) in a bucket and taking subsamples for water

analysis. Typically a volume of 7 Liters is needed. Samples should be transported to the laboratory as cool and as quickly as possible.

In addition the following environmental parameters useful for interpretation of the occurrence of cyanobacteria in general should be recorded: Light availability in the water-column, Secchi depth and temperature profiles to determine mixing depth (Wetzel & Likens 2000, exercise 2).

3) Water analysis

Both the net sample and the quantitative integrated sample will be subdivided into the following aliquots: (1) sample for cell microscopic counting; (2) sample for genotype quantification analysis per volume of filtered water; (3) sample for the isolation of single live colonies/filaments and genotype quantification on an individual basis

For cell microscopic counting phytoplankton samples from both net sampling and integrated sampling need to be conserved using the Lugol's fixative: dissolve 10g I₂ (pure iodine, toxic) and 20g KI (potassium iodide) in 200 ml distilled water and 20ml concentrated glacial acetic acid. Store in ground glass-stoppered, darkened bottle (Wetzel & Likens 2000, pp171). Use about 1 ml of Lugol's iodine to preserve 100 ml of phytoplankton sample (or 3 drops for 20 ml). The resulting sample should be the colour of whisky. Samples must be protected from light because that degrades Lugol's solution. Either use brown glass bottles or store in the dark.

For DNA analysis several aliquots of samples are filtered onto glass fibre filters or membrane filters until a green colour on the filter is obtained. The vacuum filtration pressure should not exceed 0.4 mbar. For quantitative analysis the volume of water needs to be recorded and the filter is folded inside up and stored at -20°C. It has been decided within the consortium that the ISO standard (1992b) on spectrometric determination of the chlorophyll a concentration in water is applicable for filtration in DNA analysis as well. According to this standard glass fibre filters free of organic binders and retaining all particles >1 µm are recommended. For example Whatman GF/C filters (Kent, Great Britain) or GF52 or GF6 from Schleicher & Schuell (Dassel, Germany) are considered useful. Aliquots for the isolation of single colonies/filaments should be stored cool and in the dark.

Water analysis of the integrated sample only should include chlorophyll a and total phosphorus. For the analysis of chlorophyll a and total phosphorus ISO standards are available and should be used (ISO 1992 a,b). In order to provide information on the limiting

nutrient additional nutrient parameters may include dissolved nutrients, ortho phosphate (ISO 1998), nitrate (ISO 1992a), ammonia (DIN 1983) .

For the determination of cell numbers samples are enumerated by means of an inverted microscope using the methods of Utermöhl (1958) typically one month after samples have become fixed with Lugol's solution. Cells are counted in transects of a chamber filled with a few milliliters for sedimentation over night (see Lawton et al. 1999 for details). Back-calculating to a ml of sample requires the volume of the counting chamber and measuring the area of the transects and of the chamber bottom. In order to obtain an accurate estimation of cell numbers of *Microcystis* the colonies are disintegrated by ultrasonication prior to counting (200 cycles for 0.4 s (= 4 min) in 10 ml sample). Pilot experiments with laboratory strains via microscopical counting of cells revealed no lysis of cells at sonication up to 4 min. Since *Planktothrix* and *Anabaena* are growing as filaments which are more variable in length it is more accurate to estimate the length of the filaments within the boundaries of the counting grid. At least 400 specimens of the few dominant phytoplankton species are counted at 400x magnification and the results were averaged from at least two transects per sedimentation chamber.

4) PCR analysis of single filaments/colonies

Introduction

The cyanopeptide producing cyanobacteria grow either as colonies (*Microcystis* sp.) or filaments (*Planktothrix* sp., *Anabaena* spp.). In *Microcystis* sp. the cell division process is accompanied by mucilage production, embedding the cells in a gel like matrix and one colony of *Microcystis* sp. is considered a clonal unit. There may be uncertainties about the clonality of the colonies, e.g. they may be derived from aggregation of cells originating from different genotypes. However, two pilot studies have substantiated the hypothesis of clonality in *Microcystis* sp. (Fastner et al. 2001, Kurmayer et al. 2002, see Fig.1). The assumption that single filaments represent a multicellular clonal organism is generally accepted (Hayes et al. 2002).

In recent years single filament analysis or colonies via PCR has significantly advanced in the field of molecular ecology of cyanobacteria. Walsby and co-workers have used direct lysis of

single filaments in PCR buffer and subsequent PCR amplification of one or several gene loci (reviewed in Hayes *et al.* 2002). This technique has been used for the analysis of genetic diversity among different genes, i.e. PC-IGS, rDNA-ITS, *gvpA/C* known to occur in every genotype and standardization of success/failure of PCR is usually based on the number of PCR products obtained from a specific gene locus. However, to investigate the patchy distribution of *mcy* genes among closely related genotypes it is necessary to use standardization independent of the distribution of *mcy*, i.e., via the amplification of PC-IGS. This technique has been successfully introduced for the analysis of *mcy* distribution among individual colonies of *Microcystis* sp. (Kurmayer *et al.* 2002). Since the mucilage is composed of polysaccharides the colonies typically disintegrate in Millipore water and one aliquot is used as a template for the PCR amplification of PC-IGS to achieve standardization of the success/failure of PCR. In pilot experiments, the filaments of *P. rubescens* did not disintegrate in Millipore water, or in PCR buffer and an additional ultra sonification step was needed to achieve the homogeneous disintegration of the filaments. The success rate of this improved technique for the single filament analysis of *P. rubescens* (72%, Kurmayer *et al.* submitted) was similar to the success rate obtained by Beard *et al.* (1999), i.e., 80% for investigating the diversity of *gvp* in *P. rubescens* from Lake Zürich.

Protocol for the isolation of single colonies/filaments

- 1) Take a vertical net haul using a plankton net (40µm mesh size) and keep the samples cool and protected from direct sunlight during transport to the laboratory.
- 2) For colony/filament isolation samples are diluted with nutrient medium, e.g. BG 11 (Rippka 1988) and individual colonies picked out by the means of tiny Pasteur pipettes or forceps under a binocular microscope. Colonies/filaments are washed in BG11 medium to eliminate other colonies/filaments or cyanobacteria.
- 3) Using the microscope the morphological characteristics (for colonies see Komarek & Anagnostidis 1999, see Table 1 in Via Ordorika *et al.* submitted, for *Planktothrix* see Komarek 2003), the colony/filament size (the largest diameter) and the cell size (at 400 fold magnification) are determined. To avoid squashing of the colony/filament no coverslip should be used.
- 4) The colonies are transferred into a reaction tube containing Millipore water or culture medium (final sample volume 10µl) and the presence of each colony in the tube may be verified microscopically. The tubes are stored frozen at -20°C. Thawing and

freezing several times improves disintegration of the colonies. Filaments of *Planktothrix* are mechanically disrupted by a sonicator for 10 seconds (output 40) and centrifuged for 1 min at 10,000g.

For PCR analysis of colonies/filaments 1 μl of a sample is subsequently incubated into reaction tubes for PCR. Each sample is analyzed for the PC-IGS region (the intergenic spacer region within the phycocyanin operon, Neilan *et al.* 1995) and a region within the *mcy* gene cluster encoding microcystin synthesis in parallel (see Table 1). Except of the PC-IGS primers designed by Neilan et al. 1995 all primers have been proven specific for the species and the gene region under investigation. The amplification of the PC-IGS region is used as a reference to standardize the PCR and all colonies/filaments that failed to give a PCR product of PC-IGS are omitted from further analysis. Filaments yielding no PCR product for *mcy* are tested up to three times. PCR amplifications are performed in a volume of 20 μl , containing 2 μl of Qiagen PCR buffer, 1.2 μl MgCl_2 (25 mM, Qiagen), 0.6 μl deoxynucleotide triphosphates (10 μM each, MBI Fermentas, St. Leon-Rot, Germany), 1 μl of each primer (10 $\text{pmol } \mu\text{l}^{-1}$), 0.1 μl Taq DNA polymerase (Qiagen), 13.1 μl sterile Millipore water and 1.0 μl of the sample. The PCR thermal cycling protocol includes an initial denaturation at 94°C for 3 min, followed by 35 cycles (colonies) or 40 cycles (filaments) at 94°C for 30 s, an annealing temperature of 52°C for 30 s, and elongation at 72°C for 0.5 min. PCR products are analysed by electrophoresis in 1%-1.5% agarose in 0.5x TBE (Tris-borate-EDTA) buffer and visualized by ethidium-bromide staining.

Table 1: Oligonucleotide primers used for PCR amplification of the phycocyanin *cpcB-cpcA* intergenic spacer region and of the *mcy* gene cluster encoding microcystin biosynthesis. References: (1) Kurmayer et al. 2002, (2) Hisbergues et al. (2003), (3) Kurmayer et al. (2003), (4) Kurmayer & Kutzenberger (2003), (5) Via Ordorika et al. Submitted, (6) Kurmayer unpublished, (7) Vaitomaa et al. (2003); species: *Anabaena* (A), *Microcystis* (M), *Planktothrix* (P)

Primer	Sequence (5' to 3')	T _m (°C)	Direction	Amplified product (bp)	Technique	Species	Reference
<i>cpcB-cpcA</i>							
PCβF	GGCTGCTTGTTTACGCGACA	62	F	<i>cpcB-cpcA</i> (685)	Single colony analysis	M	1,5
PCαR	CCAGTACCACCAGCAACTAA	60	R				
PcPI+	TGCTGTGCGCTAATTTTTCA	51.2	F	<i>cpcB-cpcA</i> (271)	Single filament analysis	P	6
PcPI-	CCACTGATCAGGCTGTCAGA	50.6	R				
PcMafwd	GGTCTGCGCGAAACCTATGT	62.4	F	<i>cpcB-cpcA</i> (368)	PCR dilution assay	M	3
PcMarev	GGTCAACACTTTAGCGGCG	61.6	R				
188F	GCTACTTCGACCGCGCC	54.1	F	<i>cpcB-cpcA</i> (66)	Real-time PCR	M	4
254R	TCCTACGGTTTAATTGAGACTAGCC	54.3	R				
TaqmanMaPC	FAM-CCGCTGCTGTCGCCTAGTCCCTG-TAMRA	65.7	F				
PIPe11F	GCAGGAATTACTCCTGGAGATTGT	54.6	F	<i>CpcB-cpcA</i> (71)	Real-time PCR	P	6
PIPe81R	GCCGCAGCGAGATCAAAG	54.2	R				
PIPe37T	FAM- CGCTCTGGCTTCTGAAGTCGCCG-TAMRA	65.9	F				
<i>mcy</i>							
tox4f	GGATATCCTCTCAGATTCCGG	57	F	<i>mcyBA1</i> (1312)	Single colony analysis	M	1,5
tox4r	CACTAACCCCTATTTTGGATAACC	59	R				
McyA-Cd1F	AAAATTA AAAAGCCGTATCAAA		F	<i>McyA</i> (291)	Single colony analysis	M	2,5
McyA-Cd1R	AAAAGTGTTTTATTAGCGGCTCAT		R				
peamso+	ATCAAACAGATGTACTGACAGGT	47.2	F	<i>mcyA</i> (174)	Single filament analysis	P	6
peamso-	AGGCCAGACTATCCCGTT	48.3	R				
McyBMafwd1	AATCAACGGTTAGTTGCTTATGT	56.8	F	<i>mcyB</i> (288)	PCR dilution assay	M	3
McyB-	CACTAACCCCTATTTTGGATAACC	57.7	R				
30F	CCTACCGAGCGCTTGGG	53.9	F	<i>mcyB</i> (78)	Real-time PCR	M	4
108R	GAAAATCCCCTAAAGATTCCTGAGT	54.5	R				
Blau53T	FAM-CACCAAAGAAACACCCGAATCTGAGAGG-TAMRA	64.5	F				
PL- <i>mcyBA18F</i>	ATTGCCGTTATCTCAAGCGAG	53.7	F	<i>mcyB</i> (76)	Real-time PCR	P	6
PL- <i>mcyBA183R</i>	TGCTGAAAAAACTGCTGCATTAA	54.6	R				

PL- mcyBA130T	FAM-TCAGAGGAAAGAGCTTCACCTCCACAAAAA-TAMRA	64.9	F				
mcyE-F2	GAAATTTGTGTAGAAGGTGC		F	McyE	Real-time PCR	A,M	7
MicmcyE-R8	CAATGGGAGCATAACGAG		R			M	
AnamcyE-12R	CAATCTCGGTATAGCGGC		R			A	

Validation of results

To control for biases either due to the contamination of single cells originating from other genotypes or the aggregation of two genotypes, a number of colonies isolated from Lake Wannsee was split in half and each parallel was analysed separately (Kurmayer et al. 2002). For non-*mcy* genotypes all parallels of 31 tested colonies gave identical results, for *mcy* genotypes all but five parallels of 27 tested colonies gave identical results (Fig. 2). The results of these five colonies may be either due to contamination of single cells or the aggregation of *mcy*-genotypes with non-*mcy*-genotypes. In either case the bias did not exceed 10%.

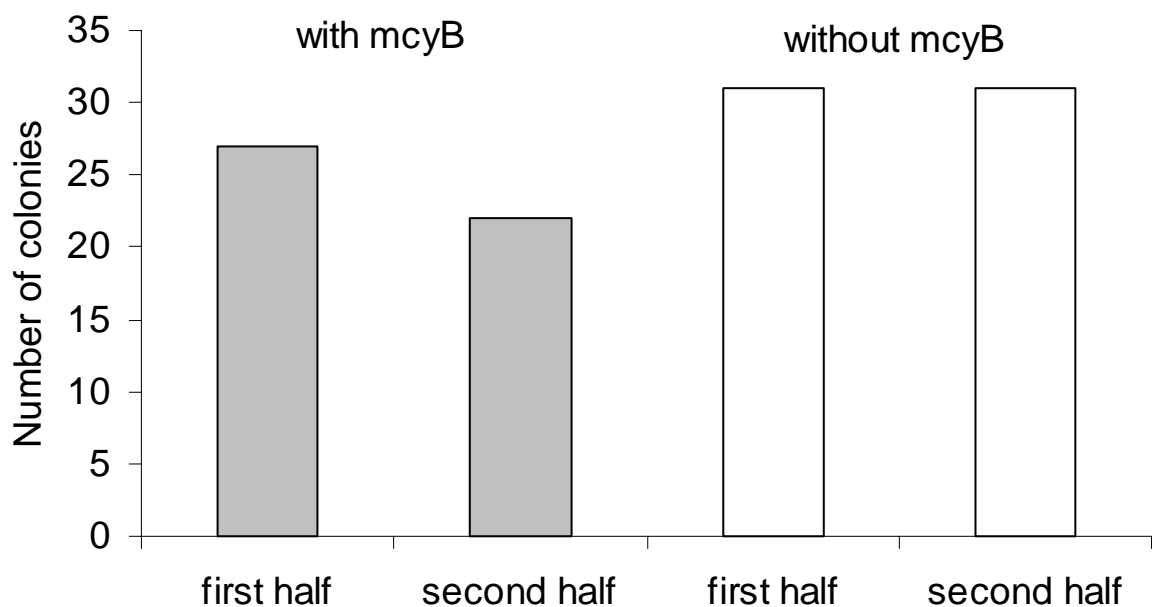


Fig. 2: Splitting colonies in half to test for reliability of PCR results in single colony analysis of *Microcystis* sp. From either *mcyB* genotypes or non-*mcyB* genotypes each parallel was analysed separately. From Kurmayer et al. (2002).

In the study of Via Ordorika et al. (submitted) a mismatch between results obtained from *mcyB* and *mcyA* primers for colonies of *Microcystis* sp. was observed. Analysis of 224 colonies for both *mcyB* by PCR and microcystin by matrix assisted ionisation time of flight mass spectrometry (MALDI-TOF MS) showed that a considerable proportion (48 of a total of 128) of microcystin containing colonies did not give a PCR product for *mcyB*. These 48 colonies were subsequently tested for *mcyA*. 42 of

these colonies were found to contain *mcyA*, however, six colonies did not give a PCR product for either the *mcyB* or the *mcyA* gene. The *mcyBA1* primers exactly match the corresponding gene region of all eight *mcyBA1* sequences currently available in the EMBL/GenBank database (October 2003). A factor accounting for the higher number of false negatives using amplification of *mcyB* when compared to *mcyA* might be its larger amplicon size: generally amplification efficiency is higher for smaller PCR products, resulting in higher sensitivity when compared to larger amplification fragments, such as *mcyB*. Consequently, it is important that both primers (for the control gene and the *mcy* gene) exhibit the same amplification efficiency.

Size limits in colony/filament isolation

During the isolation of colonies of *Microcystis* sp. in Lake Wannsee it was found impossible to discriminate between colonies <100 μm according to morphological criteria (Kurmayer et al. 2002). All colonies were in the size range between 100 and 3600 μm diameter (mean 1150 μm). Furthermore, a relationship between colony detection and colony size was observed (Via Ordorika et al. submitted). For PCR of the PC-IGS region, the smallest colonies (< 200 μm) showed the highest drop-out rate (proportion of negative samples, Table 2). The proportion of negative samples decreased with an increase in colony size, e.g. was lowest (< 3%) for colonies larger than 400 μm with PCR analysis.

A similar relationship between filament length and successful PCR amplification was observed when analyzing 252 filaments of *Planktothrix rubescens* from various lakes (Kurmayer unpublished, Table 3). The shortest filaments (<400 μm) had the lowest proportion of successful PCR amplification (65%). The percentage increased with filament length and was ca. 90% for filaments >1400 μm . In total, 71 % of the filaments were positive for PC-IGS.

Table 2: Number of colonies analysed and ‘drop-out rates’, i.e. number (percentage) of negative samples during PCR (PC-IGS) analysis of individual colonies with different colony size. Samples giving no PCR product were tested three times (from Via Ordorika et al. submitted).

Colony size (μm)	Number of colonies	Drop-out rates	
		no PC-IGS	PCR signal
<200	18	3 (17%)	
201-400	71	7 (10%)	
401-600	47	1 (2%)	
601-800	37	1 (3%)	
801-1100	48	3 (6%)	
>1101	43	1 (2%)	
unknown size	58	1 (2%)	
Total	322	17 (5%)	

Table 3. Length distribution of individual filaments sampled from various field populations of *P. rubescens* analyzed by PCR and proportion of those containing the phycocyanin (PC-IGS) and the *mcyA* gene (Kurmayer unpublished).

Filament length (μm)	Filament number	PC %
<200	23	65
201-400	59	64
401-600	53	68
601-800	36	69
801-1000	23	78
1001-1400	28	75
1401-1800	13	92
>1800	17	88
Total	252	71

5) Application of the PCR dilution assay

Introduction

The approach of colony/filament isolation cannot be directly applied for the genetic analysis of the total population in the field, because it is limited to colonies/filaments

sufficiently large for their individual selection and morphospecies determination, and also because it is too time-intensive for application to a larger number of field samples. A quantitative approach suitable for characterizing genotype composition in entire field populations was developed by Kurmayer et al. (2003). To quantify the proportion of microcystin-producing genotypes, the DNA of a sample is diluted by a series of dilutions and each dilution step is analysed by PCR for the presence of the *mcyB* gene region indicative of microcystin production. It is hypothesized that if a specific size class is composed solely of microcystin-producing genotypes it should not be possible to dilute out the microcystin gene at a higher DNA concentration than a control gene. In contrast, if the proportion of microcystin-producing genotypes is low, then the microcystin gene will be diluted out some dilution steps before the control gene. At a given amount of DNA, the difference in the number of PCR products between the two gene regions for each dilution series can be taken as a measure for the proportion of microcystin-producing genotypes.

DNA extraction

For DNA extraction defined volumes of water samples are filtered using low vacuum pressure (<0.4 bar) onto glass fibre filters (GF/C, Whatman, Kent, Great Britain). The filters are stored frozen (-20°C) or dried in a Speed Vac. The frozen filters are extracted using a protocol following Franche & Damerval (Franche & Damerval 1988). The modified protocol has been published in Kurmayer et al. (2003).

1. Cut the frozen filter into pieces and keep on ice.
2. Split the filter in half using two reaction tubes (2ml) if the diameter > 4.7 cm and suspend filter in 0.5 ml TES and incubate on ice (2h). The DNA extract is combined later. TES: 25% w/v saccharose, 50 mM Tris-HCl, 100 mM EDTA, pH 8.0.
3. Add lysozyme (~ 5mg/ml) and incubate 1h at 37°C
4. Add proteinase K (~ 50 µg/ml) and SDS (final concentration 2%), 55 µl 20% SDS and incubate 1h at 60°C
5. Add Roti phenol-chloroform-isoamylalcohol (from Roth) (25:24:1, v/v) 1 ml and vortex

6. Centrifuge 10 min 14000 rpm, and suck clear supernatant (water phase with DNA) with a pipette and put it into a new tube, don't take the white interphase (proteins)
7. Add 1 ml Roti phenol-chloroform/isoamylalcohol to the supernatant once again, vortex, centrifuge and suck the water phase again
8. Add 1 ml chloroform-isoamylalcohol (24:1, v/v) to the water phase collected in step 6 and centrifuge
9. Suck the water phase for a last time and add 2.5 volumina of ethanol (-20 °C) (ca. 1.3 ml) and precipitate DNA 1h at 0°C
10. Centrifuge 20 min at 13000 rpm, keep the pellet and discard the supernatant, the pellet is strong and the reaction tubes can be easily turned around and get the last drop with a paper,
11. Wash with 0.5 ml 70% ethanol (-20°C) and centrifuge 20 min at 13000 rpm (4°C), then suck supernatant with a pipette, the pellet is more loosely now
12. air dry the pellet, resuspend the pellet in 50 µl Millipore water

PCR Dilution assay

1. Dilute the extracts to a standard DNA concentration equivalent to 200 cells μl^{-1} as inferred from cell numbers of *Microcystis* determined from Lugol counting.
2. This standard dilution is then diluted up to 1000 fold through 14 dilution steps (the dilutions were by factors of 2, 6, 10, 14, 20, 25, 33, 50, 100, 200, 300, 400, 500, 1000 corresponding to 100, 33, 20, 14, 10, 8, 6, 4, 2, 1, 0.7, 0.5, 0.4, 0.2 cells, respectively).
3. Analyze each dilution series by PCR in the same thermal block of the cyclor for the presence of the two gene regions (PC, *mcyB*) simultaneously following PCR protocol given below.
4. For analysis the number of PCR products (the number of bands on the gel) obtained in the dilution series for *mcyB* is divided by the number of bands obtained in the dilution series for PC and a *mcyB*/PC ratio is calculated. All dilution steps yielding a PC product but no *mcyB* product are considered as DNA extracted from non-microcystin-producing cells (genotypes).

PCR amplifications are performed in a volume of 20 μ l, containing 2 μ l of Qiagen PCR buffer (Qiagen, VWR International, Austria), 1.2 μ l $MgCl_2$ (25 mM, Qiagen), 0.6 μ l deoxynucleotide triphosphates (10 μ M each, MBI Fermentas, St. Leon-Rot, Germany), 1 μ l of each primer (10 pmol), 0.1 μ l Taq DNA polymerase (Qiagen), 13.6 μ l sterile Millipore water and 0.5 μ l of the sample. The PCR thermal cycling protocol includes an initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, at an annealing temperature of 50°C for 30 s, and at 72°C for 0.5 min. PCR products (4 μ l of the reaction mix) are visualized by ethidium-bromide staining and by electrophoresis in 1.5 % agarose in 0.5 \times TBE-buffer.

The sequences for the PC primers were designed from the highly variable intergenic spacer region within the phycocyanin operon (Neilan et al. 1995) and showed sufficient specificity for the genus *Microcystis*. According to tests with a number of isolates no reactions with corresponding gene regions of other genera from cyanobacteria (e.g. *Planktothrix*, *Aphanizomenon*, etc.) have been observed neither for the PC primers nor for the *mcyB* region (data not shown). Both primer pairs have been tested for the successful amplification of the DNA originating from various isolates from Lake Wannsee (isolates W75, W334, W368 as reported by Rohrlack et al. (2001) as well as the restriction fragment length polymorphism types I and II (the two most significantly different groups of genotypes reported in Kurmayer et al. 2002.

Validation of results

To test the specificity and sensitivity of the PCR dilution assay under natural conditions the microcystin-producing strain HUB 5-2-4 was grown in batch culture in Z medium (Zehnder & Gorham 1960) and 5 ml of a cell concentration of 2.08×10^7 cells ml^{-1} (determined by a Casy $\text{\textcircled{R}}$ 1, Schärfe system, Reutlingen, Germany) were filtered on a GF/C-filter and the DNA extracted as described above. From the extract five dilutions ranging from 1:10² to 1:10⁶ of template DNA (equivalent to 10,400 cells to 1.04 cells) for PCR were prepared and analysed by PCR with both primer pairs in the absence or presence of a 1:100 dilution of a natural background. To obtain the natural background without presence of microcystin-producing *Microcystis* genotypes, water from Lake Wannsee was filtered on 4th May 2000 through a sieve (25 μ m mesh size) and 300 ml were filtered on a GF/C filter and the DNA extracted.

The counting of cyanobacteria under the inverted microscope (3.2 ml sedimentation chamber) revealed the dominance of *Aphanizomenon* spp., *Limnothrix* spp., *Limnothrix redekei*, *Planktothrix agardhii* with a biovolume of 0.38, 1.95, 1.11, 1.0 mm³ L⁻¹, respectively (unpublished data). No cells of *Microcystis* were found after careful examination of two transects of the counting chamber at 400-fold magnification. Both primer pairs showed high specificity and identical sensitivity within the range of 10,400 to 1.04 cells as DNA template in the absence and presence of the background (Fig.3).

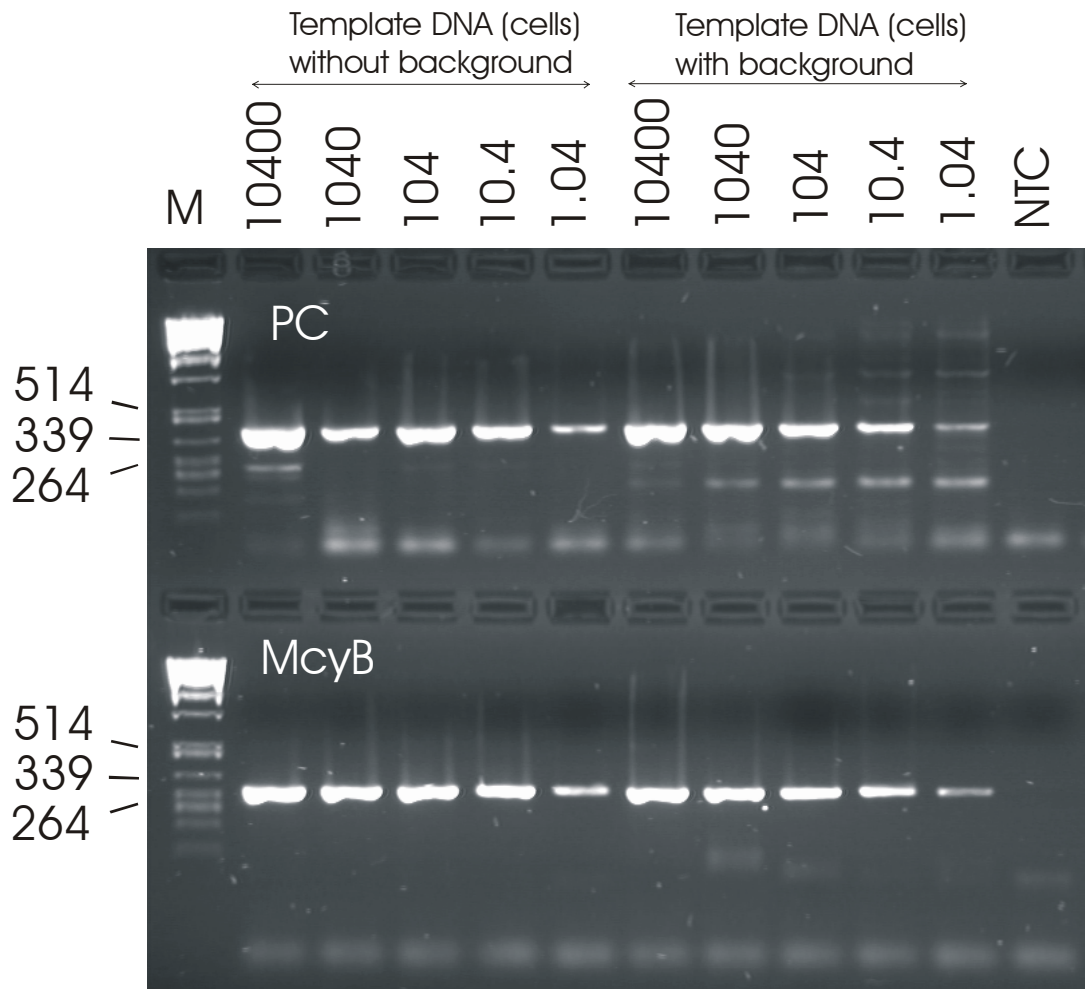


Fig. 3: Photograph of an ethidium-bromide stained gel showing the amplification products of the phycocyanin internal transcribed spacer region (PC) and of a microcystin specific region (*mcyB*) for a dilution series of the microcystin-producing strain *Microcystis* sp. HUB 5-2-4. The numbers refer to template DNA (equivalent to 10,400 cells to 1.04 cells) in the presence/absence of a natural background from Lake Wannsee. For details on the preparation of the natural background see text. M = molecular weight marker in base pairs, NTC = non-template negative control. From Kurmayer et al. (2003).

The validity of the PCR dilution assay was further assessed using two *Microcystis* strains, HUB 5-2-4 (*mcyB*) and HUB 5-3 (non-*mcyB*). DNA extracts equivalent to 200 cells μl^{-1} either originating from strain HUB 5-2-4 (*mcyB*) or from strain HUB 5-3 (non-*mcyB*) were mixed at ratios with 0.1 %, 1.0 %, 10 %, 100% of HUB 5-2-4 and then analyzed by the dilution assay as described above. The total number of PC products obtained by PCR was stable for the full range of the *mcyB*/non-*mcyB* mixture of the DNA template for three independent measurements (Fig. 4A). In contrast, the total number of *mcyB* products decreased accordingly with the reduction in the proportion of the *mcyB* strain in the DNA template. Consequently, the ratio of *mcyB*/PC products showed a significant linear positive relationship with the percentage of the *mcyB* strain in the DNA extract (Fig. 4B).

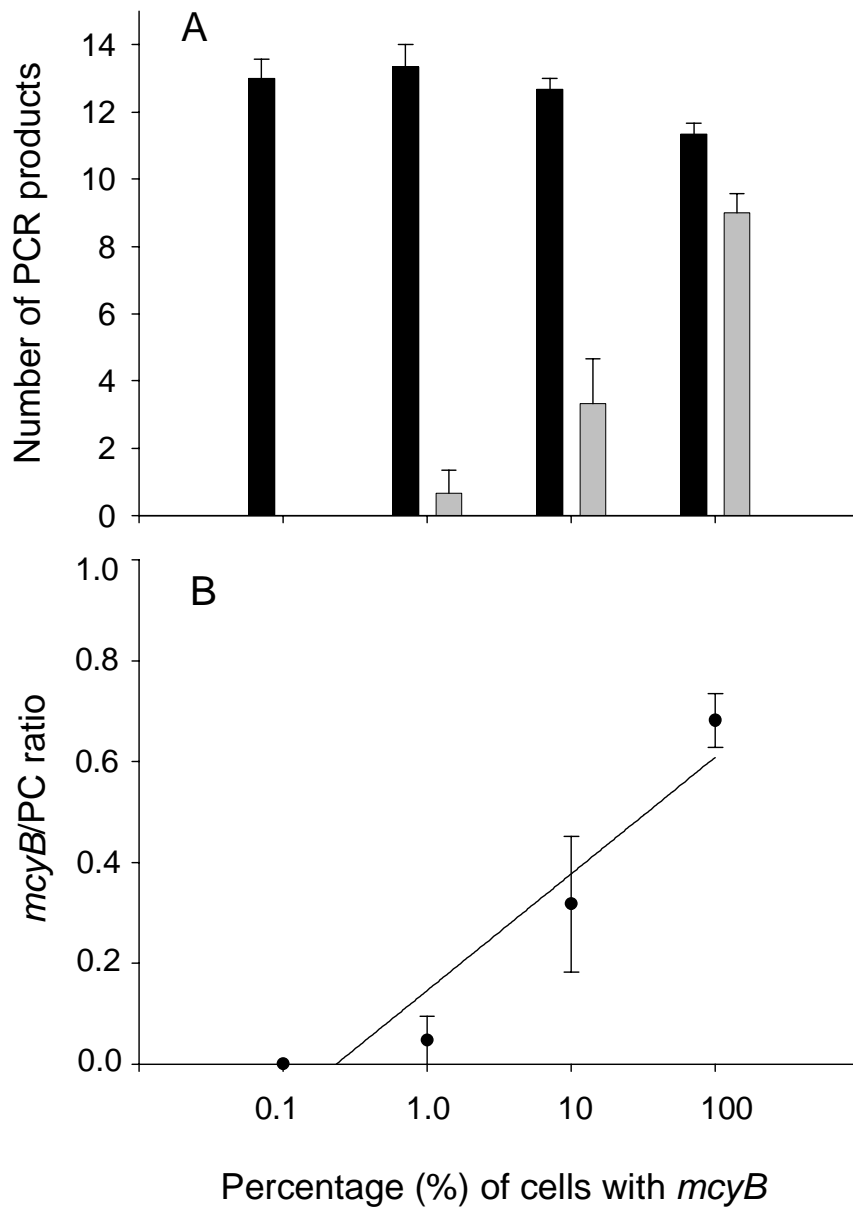


Fig. 4: (A) Total number of PC (phycocyanin, black columns) and *mcyB* (microcystin, white columns) products obtained by PCR in a dilution assay at various proportions of DNA from *mcyB* cells in a DNA extract prepared from non-*mcyB* cells. For the details on the dilution assay see text. (B) The ratio of *mcyB* products to PC products obtained by PCR in the dilution assay was found to be linearly related to the proportion of DNA from *mcyB* cells ($y = 0.075 + 0.007x$, $R^2 = 0.95$, $n = 12$, where $y =$ *mcyB*/PC ratio, $x =$ percentage of *mcyB* cells). From Kurmayer et al. (2003).

6) Quantification of genotypes using real-time PCR

Introduction

The real-time PCR system is based on the detection and quantification of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The threshold cycle or the C_t value is the cycle at which a significant increase in fluorescence signal is first detected. The threshold cycle is when the system begins to detect the increase in the signal associated with an exponential growth of PCR product during the log-linear phase. This phase provides the most useful information about the reaction (certainly more important than the end point). The slope of the log-linear phase is a reflection of the amplification efficiency. For the slope to be an indicator of real amplification (rather than signal drift), there has to be an inflection point. This is the point on the growth curve when the log-linear phase begins. It also represents the greatest rate of change along the growth curve. (Signal drift is characterized by gradual increase or decrease in fluorescence without amplification of the product.) The important parameter for quantification is the C_t . The higher the initial amount of genomic DNA, the sooner accumulated product is detected in the PCR process, and the lower the C_t value. The choice of threshold, which will determine the C_t value is up to the operator and one of the subjective elements in real-time PCR. It should be placed above any baseline activity and within the exponential increase phase (which looks linear in the log transformation).

There are two general methods for the quantitative detection of the amplicon: (1) DNA-binding agents and (2) fluorescent probes. The most commonly used technique is the double-stranded DNA binding dye chemistry, which quantifies the amplicon production (including non-specific amplification and primer-dimer complex) by the use of a non-sequence specific fluorescent intercalating agent (i.e. SYBR-green I). SYBR Green I is a minor groove binding dye. It does not bind to ssDNA. The major problem with SYBR Green-based detection is that non-specific amplifications cannot be distinguished from specific amplifications. Melting-curve analysis can be used to

determine specificity, where the temperature is raised slowly to the melting point of the amplification product and fluorescence is monitored. Since SYBR Green I only binds double-stranded DNA, the fluorescence signal decreases as the melting temperature (T_m) of the DNA duplex is reached. Analysis of the melting curve allows confirmation of PCR products.

The TaqMan probes use the fluorogenic 5' exonuclease activity of Taq polymerase to measure the amount of target sequences (Heid et al. 1996). TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a T_m value of 10 °C higher) that contain a fluorescent dye usually on the 5' base (FAM), and a quenching dye (usually TAMRA) typically on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = Förster or fluorescence resonance energy transfer). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase replicates a template on which a TaqMan probe is bound, its 5' exonuclease activity cleaves the probe. This ends the activity of quencher (no FRET) and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). TaqMan assay uses universal thermal cycling parameters and PCR reaction conditions. Because the cleavage occurs only if the probe hybridizes to the target, the fluorescence detected originates from specific amplification. The process of hybridization and cleavage does not interfere with the exponential accumulation of the product. One specific requirement for fluorogenic probes is that there is no G at the 5' end. A 'G' adjacent to the reporter dye quenches reporter fluorescence even after cleavage.

SYBR Green I assay

Two genera, *Anabaena* and *Microcystis* are known to produce microcystins and may occur in equal abundance in lakes of the temperate region. Vaitomaa et al. (2003) have used real time PCR to find out and quantify the main microcystin-producers in lakes inhabiting both genera. Genus specific *mcyE* primers were designed from *mcyE* encoding the glutamate-activating adenylation domain (Table 1) and used to quantify

Microcystis and *Anabaena mcyE* copy numbers occurring in two Finnish lakes. External standards used to determine *mcyE* copy numbers were prepared using genomic DNAs of *Microcystis* strains and *Anabaena* strains. Approximate genome sizes have been used in the *mcyE* copy number calculation (see Vaitomaa et al. 2003 for details). Linear regression equations for obtained cycle threshold values (C_t) were calculated as a function of known *mcyE* copy numbers.

Protocol

- 1) Extract and purify DNAs from lake water samples (details in Giovannoni et al. 1990). DNA extracts are purified with a Prep-A-gene DNA purification Kit (Bio-Rad) according to the manufacturers instructions and eluted in 60 μ l.
- 2) Reactions are performed with 1 μ l of DNA from a standard strain or lake water sample, 3 mM $MgCl_2$, 0.5 μ M concentrations of both primers and 1 μ l of hot start reaction mix to a final volume of 10 μ l. An initial preheating step of 10 min at 95°C was followed by 45 cycles, with 1 cycle consisting of 2 s at 95°C, 5 s at 58°C, and 10 s at 72°C. Generation of the PCR products was monitored after each extension step at 78°C in *Microcystis* and 77°C in *Anabaena* by measuring SYBR green 1 dye created fluorescence. In order to determine melting temperatures for the PCR products, the temperature was raised after cycling from 65 to 95°C, and fluorescence was detected continuously. All external standards and lake water samples are analyzed in triplicate.
- 3) Copy numbers of *mcyE* genes are determined by converting the obtained C_t values into *mcyE* copy numbers according to the regression equations obtained with external standards:

Taq nuclease assay

Recently, the TaqMan PCR or the Taq nuclease assay has been introduced to quantify specific genotypes of picocyanobacteria (Becker et al. 2000) or microcystin-producing cyanobacteria in the field (Foulds et al. 2002). This technique uses a sequence specific dual-labelled fluorescent probe (TaqMan probe) and primers to quantify the level of DNA template initially present in a sample. The rate of

exponential accumulation of the amplicon is monitored by the hydrolysis of the TaqMan probe generating a fluorescent signal during the amplification process. The threshold cycle (C_t) is the PCR cycle number at which the fluorescence passes a set threshold level and can be used to determine the starting DNA amount in the sample based on a standard curve (based on samples with a known concentration). Using standard curves by relating cell concentrations to the threshold cycle it is possible to quantify genotypes in absolute terms, e.g. cells of microcystin genotypes in a given volume of lake water.

In the study of Kurmayer & Kutzenberger (2003) two independent Taq nuclease assays (TNA), one to quantify the total population of *Microcystis* sp. using the intergenic spacer region within the phycocyanin (PC) operon and the other assay to quantify microcystin genotypes using a region of *mcyB* part of the microcystin synthetase gene cluster were developed. A variable gene region of PC was selected based on an alignment (ClustalW 1.8) of phycocyanin genes from several genera of cyanobacteria from the GenBank including *Microcystis*, *Planktothrix* and other genera (see Kurmayer & Kutzenberger 2003 for details). The *mcyB* gene region was selected from *Microcystis* strain HUB524 (Z28338) and was located between core motifs A2 and A3 (Marahiel et al. 1997). From those gene regions optimal primers and TaqMan probes were designed using the Primer Express 2.0 software (Applied Biosystems, Vienna, Austria, Table 1). The TaqMan probes each have a fluorescent reporter dye (6-carboxyfluorescein) covalently attached to the 5' end (5'- FAM) and a 3'- TAMRA fluorescent quencher dye (6-carboxytetramethylrhodamine).

Establishing calibration curves

Standard curves relating cell concentrations to the threshold cycle are required (i) to quantify the target genotype in equivalents of cell numbers and (ii) to determine the specificity and sensitivity of the Taq nuclease assay. For both genes (PC and *mcyB*) standard curves based on pre-determined cell concentrations are established by relating the known DNA concentrations (in cell equivalents) to the threshold cycle of the diluted samples.

- 1) A pure culture of *Microcystis* sp. is grown non-axenically under sterile conditions in batch culture in a nutrient medium (e.g. according to Zehnder & Gorham, 1960) and harvested during the stationary growth phase. Cells are harvested by filtering onto glass fibre filters (GF/C, Whatman, Kent, Great Britain) under vacuum pressure and stored frozen (-20°C) until DNA extraction.
- 2) Extract DNA from frozen filters as described previously in chapter ...
- 3) Aliquots are analyzed for cell numbers using at least two of three independent methods: (1) electronic particle counting (e.g. Casy® 1, Schärfe system, Reutlingen, Germany), (2) counting of cells by the inverted microscope technique (e.g. Utermöhl 1958), (3) cell counting using autofluorescence or DAPI staining (Porter & Feig 1980, because not all strains may sediment consistently in sedimentation chambers). At least 400 specimens of *Microcystis* are counted at 400x magnification and the results are averaged from at least two transects per sedimentation chamber or filter.
- 4) From the DNA extract six dilutions ranging from 1:10² to 1:×10⁷ of template DNA (equivalent to counted cells) are prepared and analyzed for both genes in the absence or presence of a background. To compare between background effects the number of cells estimated in the presence of a natural background is divided by the cell number estimated in the absence of a natural background and a ratio of cells with background/cells without background is calculated. The more the ratio deviates from one the stronger the background effects.
- 5) The background is either prepared from environmental DNA originating from the target ecosystem or from DNA of most closely related strains that do not have the target genotype, i.e. if *mcyB* is the target genotype then a non-*mcyB* strain of the same genus is used for background DNA. Typically background is added at dilution 1:100 or 1:1000 and the measurements are compared to controls without background.
- 6) PCR is initiated with two holds, one for 2 min at 50°C (AmpErase® UNG protection against carry-over contamination), followed by 10 min at 95°C. Subsequently a 95°C denaturation step for 15 s was followed by a 60°C annealing and extension step for 1 min, for 45 cycles. Reactions are performed with a volume of 25 µl, containing 12.5 µl of 2 × TaqMan® Universal PCR

Master Mix (ABI, Vienna), 300 nM (300 fmol μl^{-1}) of each primer, 100 nM of the TaqMan probe, 5 μl of template containing various amounts of genomic DNA and filled up to 25 μl with sterile Millipore water. For *mcyB* 900 nM of each primer and 250 nM of the TaqMan probe are used. Each measurement is done in triplicate.

- 7) Increased quantification errors typically occur towards both ends of the calibration curve. Consequently for field analysis cell quantification should be directed towards the central region of the standard curves (i.e. 1,000 cells) which are found to be most resistant against background effects.
- 8) Measurements on samples should be done in exactly the same way as for the measurements on samples for standard curves.

Validation of results

Comparing DNA extraction methods

To compare DNA quality obtained by the DNA extraction procedure (chapter ...) and a commercially distributed DNA extraction kit (DNeasy® Plant Mini Kit, Qiagen) aliquots of *Planktothrix* strain PCC7821 were harvested during the logarithmic growth phase via filtering on Whatman GF/C filters (2.5 cm) and the DNA extracted as described in chapter ... or following the DNeasy® Plant Mini Kit extraction protocol (Qiagen 2000). To quantify the PC-IGS gene in *Planktothrix* the primers and TaqMan probe as given in Table 1 at 900nM of each primer and 250nM of the TaqMan probe have been used. For both extracts two parallels were measured in triplicate at an dilution of 1:1000 and the treshhold cycle values were $C_t = 24.5 \pm 0.1$ (SD) for the DNA extraction kit and $C_t = 24.3 \pm 0.04$ (SD) for the standard DNA extraction procedure (Kurmayer, Schober unpublished). It is concluded that both DNA extraction techniques do give reliable qualitative and quantitative results.

Comparing DNA extraction quality from freeze dried filters and wet filters

In order to compare DNA quality obtained from wet (frozen) filters or filters that have been freeze dried aliquots of *Planktothrix* strain PCC7821 were harvested via filtering on Whatman GF/C filters and stored frozen at -20°C or dried in a vacuum centrifuge (Speed Vac, Eppendorf) at 30°C for 4 hours and stored at -20°C afterwards. From

mcyBA1 gene regions of *Planktothrix* optimal primers and a TaqMan probe were designed and used at 900nM (primers) and 250nM (Taqman probe) in reactions. For both extracts two parallels were measured in triplicate at a dilution of 1:1000 and the threshold cycle values were $C_t = 27.4 \pm 0.8$ (SD) for the freeze dried samples and $C_t = 27.5 \pm 1.8$ (SD) for the wet frozen samples (Kurmayer, Schober unpublished). Because the results do not differ it is concluded that drying of DNA samples does not influence the quality of the DNA. Storage and transport of dried samples is much easier when compared to transport of wet filters and particularly for the mailing of samples freeze drying is recommended.

7) Measuring variability in the proportion of toxic genotypes in relation to colony size in the cyanobacterium *Microcystis* sp.: comparing two independent methods

In the study of Kurmayer et al. (2003) a first attempt was made to quantify the proportion of microcystin genotypes in different colony size classes of *Microcystis* by the ratio of the number of PCR products between the microcystin gene and a reference gene obtained for a dilution series of a DNA extract. This approach revealed a general significant correlation of the frequency of the microcystin genotype proportion with colony size, i.e. the largest colonies (>100 μm) had a 10-fold higher percentage of microcystin genotypes than the smallest colonies (<50 μm). In the study of Kurmayer & Kutzenberger (2003) microcystin genotypes during seasonal development of the total population were quantified by means of real-time PCR. To validate those results of both methods it was the aim to compare the microcystin genotype proportion estimated independently by both techniques in relation to colony size.

Sampling was performed at Lake Wannsee (Berlin, Germany) from June 1999 to September 1999 and from June 2000 to October 2000. The lake is shallow (mean depth 5.5 m) and hypertrophic and regularly dominated by *Microcystis* sp. during the summer. The sieving procedure included the filtering of 15-20 L of lake water through various sieves with mesh sizes of 100 and 50 μm . The colonies larger than 340 μm were analysed separately. Each size class was analysed biweekly (PCR dilution assay) and monthly (Taq nuclease assay) for the proportion of microcystin

genotypes. Details on the filtering of samples, DNA extraction, the PCR dilution assay and the Taq nuclease assay are given in Kurmayer et al. (2003) and Kurmayer & Kutzenberger (2003).

In all size classes, the *mcyB* gene was found the whole year round. However, with the dilution assay the *mcyB* gene was quickly diluted below detection when compared to the PC gene in the smallest colony size class (<50 μm) but not in the largest size class (>340 μm). Over the whole study period the smallest size class (<50 μm) consistently revealed *mcyB*/PC ratios around 0.5 (Table 4). In contrast, the two largest size classes (>100 μm , >340 μm) consistently showed *mcyB*/PC ratios close to one ($p < 0.001$). Correspondingly, the proportion of *mcyB* genotypes as determined by the TNA was lowest within the smallest size class (<50 μm) and increased significantly in the two larger size classes ($p = 0.005$). The results of the PCR dilution assay and the TNA were found to be significantly related: $\log y = 0.20 + 1.235x$, $R^2 = 0.53$, where $y =$ *mcyB* genotype percentage as determined by TNA, $x =$ *mcyB*/PC ratio obtained by PCR dilution assay.

It is concluded that both genotype quantification methods revealed a strong gradient and a significant positive relationship with colony size. Comparing both methods is necessary because both techniques do have constraints in accuracy due to their linear-log calibration curves. Both techniques use linear-log calibration curves relating either the *mcyB*/PC ratio or the C_t values on a linear scale to the percentage of *mcyB* genotypes on a logarithmic scale. For example, using the PCR dilution assay as described above a 50% reduction in *mcyB* genotype proportion would not result in a decrease in the *mcyB*/PC ratio while a *mcyB* genotype proportion of 10% would result in a *mcyB*/PC ratio of 0.56. In addition, the TNA technique is sensitive to variations in the slope induced by minor variations of the C_t values. Consequently, the noise within C_t values induced by the semi logarithmic calibration algorithm alone can mask an increase or decrease in *mcyB* genotype proportion. However, the results of this comparison show that both techniques are able to reliably estimate variability in genotype proportions differing by a factor 10 or more. Because it has been suggested that waterbodies differ in genotype composition by a factor of 10 or more (see references in the introduction of this manual) it is believed that both techniques might

be able to identify factors that govern genotype and chemotype composition in nature.
Further studies are needed to verify this suggestion.

Table 4: Gene (*mcyB*/PC) ratios in Lake Wannsee during summer of 1999 and 2000 for each size fraction of *Microcystis*. The differences were tested using Kruskal-Wallis One Way ANOVA on Ranks followed by Dunn's multiple comparison procedure. Symbols (^{a,b}) indicate subsets whose highest and lowest medians are not significantly different ($P > 0.01$). The data are given as 25 % (percentile) - median - 75% (percentile); mean \pm C.L. (95 % confidence limit); N = sample size;

Colony size (μm)	<i>mcyB</i> /PC ratio (Dilution assay)			<i>mcyB</i> percentage (Taq nuclease assay)		
	25%-Median-75%	Mean (95% CL)	n	25%-Median-75%	Mean (95% CL)	n
<50	0.39-0.56-0.7 ^a	0.54 \pm 0.11	16	3.3-8.4-9.7 ^a	7.2 \pm 2.5	9
50-100	0.65-0.71-0.78 ^{ab}	0.76 \pm 0.12	16	7.8-9.2-15.3 ^{ab}	12.6 \pm 5.2	9
>100	0.83-0.95-1 ^b	0.97 \pm 0.13	16	16.6-26.5-41 ^b	29.4 \pm 12.3	9
>340	0.93-1-1 ^b	0.97 \pm 0.12	6	21.3-36.3-52.4 ^b	37.4 \pm 23.9	4

8) Comparing quantitative PCR results between laboratories

It has been shown in previous chapters that all real-time PCR techniques do have constraints in accuracy due to their linear-log calibration curves. Consequently, one important question was on the comparability of results achieved independently between laboratories. For this purpose a comparison of results for *Microcystis* from Lake Wannsee was performed between the Institute of Limnology, Mondsee and the Department of Applied Chemistry and Microbiology, University of Helsinki using samples taken by Kurmayer & Kutzenberger (2003), Fig. From those samples DNA extracts and primers and TaqMan probes were sent to Helsinki and the TNA assays were performed according to the protocol below.

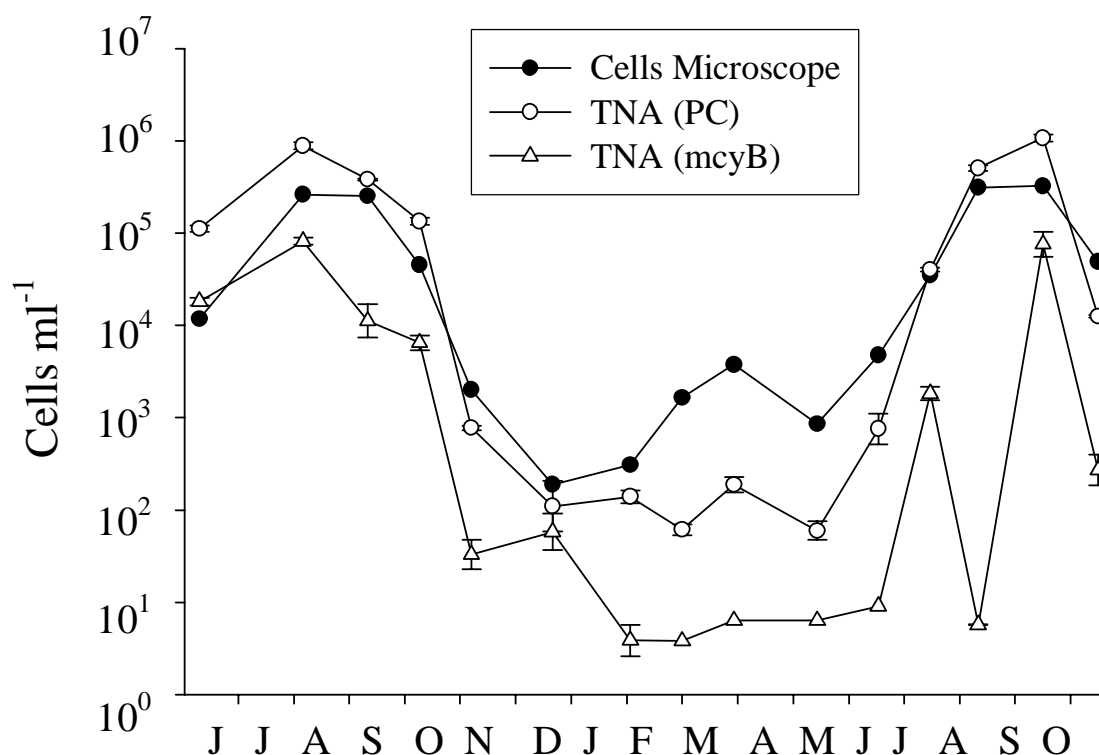


Fig. 5: Cell number of *Microcystis* in Lake Wannsee from June 1999 to October 2000 determined by counting under the inverted microscope (black circles) or by TNA via the phycocyanin gene (white circles, mean \pm 1 SE) and via the mcyB gene (triangles). From Kurmayer & Kutzenberger (2003).

The protocol contains the instructions necessary to perform two independent Taq Nuclease Assays (TNAs) for *Microcystis*, to quantify the total population (TNA for PC) and the subpopulation of microcystin-genotypes (TNA for *mcyB*) in natural waters (see also Kurmayer, R., Kutzenberger, T. (2003).

1) Storage of samples

The Wannsee-extracts and primers are stored frozen. The Taqman probe should be stored at 4 °C and protected from light, repeatedly thawing and freezing should be avoided.

2) Preparation of the measuring system

The system is switched on (we use an ABI, GeneAmp 5700), the necessary checks are made and the data are entered to label the template, the PCR protocol is rechecked and modified if necessary.

3) Preparation of samples

The DNA extracts have been prepared as described in the AEM 69, p6724 and must be diluted down to a cell concentration of 200 cells per μl of DNA extract. This concentration would result in a template of 1000 cells per reaction (we incubate 5 μl of the template) and has been shown to give most reliable results because it lies in the center of the optimal range of the calibration series ($10 - 10^4$ cells, see Fig 1A in AEM 69, p6725). Because the samples have been counted before using the inverted microscope technique we can calculate the optimal cell numbers in the template and the required dilutions. The dilution is freshly prepared for each PCR however both TNAs are performed from the same DNA extract simultaneously. We just keep the undiluted extracts frozen and this prevents possible degradation by DNA degrading enzymes over time. To reduce the inhibitory influence of extraction reagents in PCR down to a minimum, the extracts are diluted at least by a factor 100.

4) Preparation of PCR reagents (Master mix, MM)

Primers and Taqman Probes have been designed using the ABI primer express software 2.0 and ordered in a commercial oligosynthesis laboratory. The Universal MM is from ABI (Taq Man Universal PCR Master Mix - Applied Biosystems, Vienna, Austria, part number 4304437) and contains a 2 x concentration of premixed PCR buffer, Taq Polymerase, AmpErase® UNG protection against carry-over contamination.

The primer concentration is usually 100 pmol/ μl , you have to dilute them 1:10 (\rightarrow 10 pmol/ μl). The optimized primer and Taqman concentrations are as follows:

The forward primer / reverse primer / probe concentration for PC is 300/300/100 nM, for *mcyB* 900/900/250 nM. A calculation sheet (in Excel) to calculate the necessary volumes to prepare the MM is included and illustrated in **Fig. 1**. Using this sheet all necessary volumes can be easily adjusted just by entering the total number of reactions (see cell "total number of reactions = 20). One non template control (NTC) with A. dest as template is included. Each measurement is made in triplicate. The reaction volume is 25 μl (**Tab. 2**). Firstly, H₂O is pipetted, then the buffer, primers and the TaqMan probe and vortexed.

TaqMan Universal MasterMix für PC

set parameters			set optimal concentrations	
total number of reactions	20	rxns	final FP (nM) in fmol/μl	300
reaction volume	25	μl	final RP (nM) in fmol/μl	300
template volume per rxn (5-10μl)	5	μl	final P [] in nM	100
FP forward primer [5 μM]	10	μM=pmol/μl		
RP reverse primer [5 μM]	10	μM=pmol/μl		
P probe [5 μM]	40,8	μM=pmol/μl		

TaqMan Universal MasterMix (uMM) P/N 4304447 for 200 rxn. store @ 4°C	
---	--

Preparation of master mix (MM)					
reagent	concentration stock	concentration final	μl per 1 rxn	μl per rxns 20	reagent
2 x uMM Buffer	2 x	1 x	12,50	250,00	2 x uMM Buffer
FP forward primer	10	300	0,75	15,00	FP forward primer188F
RP reverse primer	10	300	0,75	15,00	RP reverse primer254R
P probe	40,8	100	0,06	1,23	P probe
template	diverse	diverse	5,00	100,00	template, add later !
water			5,94	118,77	water
total volume μl			25,00	500,00	total volume μl

volume should be	25,00	500,00
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combine	5	μl template with	20	μl MasterMix
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Fig. 6: Calculation sheet for UnivMM for the TNA for PC

Tab. 5: Reaction volumes of reagents per PCR

Volumes for one reaction:	PC	mcyB
Millipore water	5.9	2.8
buffer	12.5	12.5
primer forward [μl]	0.75 (10 pmol/μl)	2.25 (10 pmol/μl)
primer reverse [μl]	0.75 (10 pmol/μl)	2.25 (10 pmol/μl)
probe [μl]	0.06 (40,5 pmol/μl)	0.22 (28.2 pmol/μl)
DNA [μl]	5	5
Total volume [μl]	25	25

5) Set up of the reactions

The MM is aliquoted (20 μl) into optical tubes or plates and the template is added at the end. All extracts and Master mix are stored on ice during preparation of the samples. With > 50 reactions we take an optical plate, put an optical cover onto it and fasten it with the aid of the Cap Installing Tool Recorder. We place an optical cover compression pad onto the cover. The pad can be used about 20 times.

6) Temperature protocol

PCR is initiated with two holds, one for 2 min at 50°C (AmpErase® UNG protection against carry-over contamination) followed by 10 min at 95°C. Subsequently, a 95°C denaturation step for 15 s is followed by a 60°C annealing and extension step for 1 min, for 45 cycles.

Tab. 6: Temperature protocol

Temperature [°C]	length of time	number of cycles	reaction
50	2 min	1	removal of nucleid acids with uracil by Amp Erase Uracil-N-Glycosylase
95	10 min	1	activation of hot start DNA polymerase
95	15 s	45	DNA denaturation
60	1 min	45	binding of probe and primers to the template, elongation by polymerase.

7) Equipment

Tab. 7: Equipment:

Company	Product name	Part number
Applied Biosystems	Optical Tubes	N 801-0933
Applied Biosystems	Optical Caps (8 caps/strip)	4323032
Applied Biosystems	96-Well Optical Reaction Plate with Barcode (code 128)	4306737
Applied Biosystems	Optical Adhesive Covers	4311971
Applied Biosystems	Cap Installing Tool Recorder	N 801-0438
Applied Biosystems	Optical Cover Compression Pads	4312639
Applied Biosystems	TaqMan Universal PCRMaster Mix	4304437

8) Data analysis

The threshold value for the fluorescence of all samples is set manually at 0.1 in accordance with the instruction manual of the GeneAmp 5700 Sequence Detection System. Standard curves were established by relating cell concentrations to the threshold cycle (the PCR cycle number at which the fluorescence passes a set threshold level) for both TNAs and should be used. For PC and *mcvB* the regression equations are $y = 38.61 - 3.49x$ ($R^2 = 0.99$, $n = 6$, $p < 0.0001$) and $y = 46.14 - 4.07x$ ($R^2 = 0.99$, $n = 5$, $p < 0.0001$), y is the PCR cycle number (C_t) at the set fluorescence threshold level (0.1) and x is the amount of starting DNA (given as \log_{10} cell number equivalents), see AEM 69, p6725 for details on calibration curves.

Results and Discussion

In the Finnish laboratory the quantitative PCR measurements were performed with an ABI Prism 7000 sequence detection system at the Institute of Biochemistry, University of Helsinki. The ABI Prism 7000 is similar to the GeneAmp 5700 detection system. The DNA extracts during winter and spring (25 January, 22 February, 21 March, 4 May 2000) gave very flat curves only without reaching a plateau in the Helsinki laboratory. In the Mondsee laboratory these samples were below the limit of detection for *mcvB*. The standard deviations in these samples were therefore large and those samples were omitted from further analyses.

Generally, cycle of threshold (C_t) values were lower in Helsinki than in Mondsee indicating a greater sensitivity with the ABI Prism 7000 sequence detection when compared with the GeneAmp5700 system (Tab. 8). For both TNAs the correlation between the two data sets were highly significant and it is concluded that the quantitative PCR measurements are reproducible among laboratories.

Tab. 8: Cell number (cells/ml) of *Microcystis* in Lake Wannsee from June 1999 to October 2000 determined by counting under the inverted microscope or on the threshold cycle by TNA for the phycocyanin gene (PC, mean \pm 1 SE) and the mcyB gene (mcy, mean \pm 1 SE) determined in two laboratories, Mondsee (M) and Helsinki (F). Some samples (125, 114, 112 and 116) were either below the limit of detection of mcy (M) or gave very flat curves without reaching a plateau (F). Consequently, those samples were omitted from further analyses.

Date	DNA extract	Cells/ml	TNA PC M	TNA PC F	TNA mcy M	TNA mcy F
6 July 1999	142	11795	24.5 \pm 0.12	23.6 \pm 0.04	33.4 \pm 0.18	29 \pm 0.19
3 Aug 1999	134	262308	26.5 \pm 0.13	24.1 \pm 0.13	36.1 \pm 0.16	30.2 \pm 0.16
7 Sept 1999	139	252569	27.5 \pm 0.03	25.8 \pm 0.38	38 \pm 0.74	32.5 \pm 0.47
5 Oct 1999	128	45193	26.5 \pm 0.13	24.9 \pm 0.8	37.6 \pm 0.33	31.3 \pm 0.88
2 Nov 1999	119	2007	29.4 \pm 0.09	24.9 \pm 0.29	40 \pm 0.66	31.4 \pm 0.86
16 Dec 1999	122	188	29.7 \pm 0.04	28.03 \pm 0.16	38.6 \pm 0.8	34.2 \pm 0.37
25 Jan 2000	125	309	32.2 \pm 0.2	28.3 \pm 0.7 ¹	44.3 \pm 0.7	33.8 \pm 1.4 ¹
22 Feb 2000	114	1641	33.7 \pm 0.2	25.3 \pm 0.4 ¹	45 ²	24.6 \pm 1.8 ¹
21 March 2000	112	3753	33.0 \pm 0.3	27.4 \pm 0.2 ¹	45 ²	25.6 \pm 1.3 ¹
4 May 2000	116	857	33.6 \pm 0.4	29.7 \pm 0.9 ¹	45 ²	27.8 \pm 5 ¹
5 June 2000	109	4759	30.3 \pm 0.6	31.8 \pm 0.3	45 ²	38.2 \pm 0.97
4 July 2000	100	35140	28.1 \pm 0.07	25.8 \pm 0.2	39.7 \pm 0.3	32.5 \pm 0.72
1 Aug 2000	103	312976	27.5 \pm 0.1	24.7 \pm 0.1	38.3 \pm 0.19	32 \pm 0.38
5 Sept 2000	96	324301	26.1 \pm 0.13	24.3 \pm 0.06	36 \pm 0.55	27.1 \pm 0.47
4 Oct 2000	T84+T85	48800	30.3 \pm 0.05	28.5 \pm 0.2	42.6 \pm 0.67	36.3 \pm 0.36

¹... instead of a steep amplification curve a flat amplification curve was observed during analysis only.

²...below the detection limit

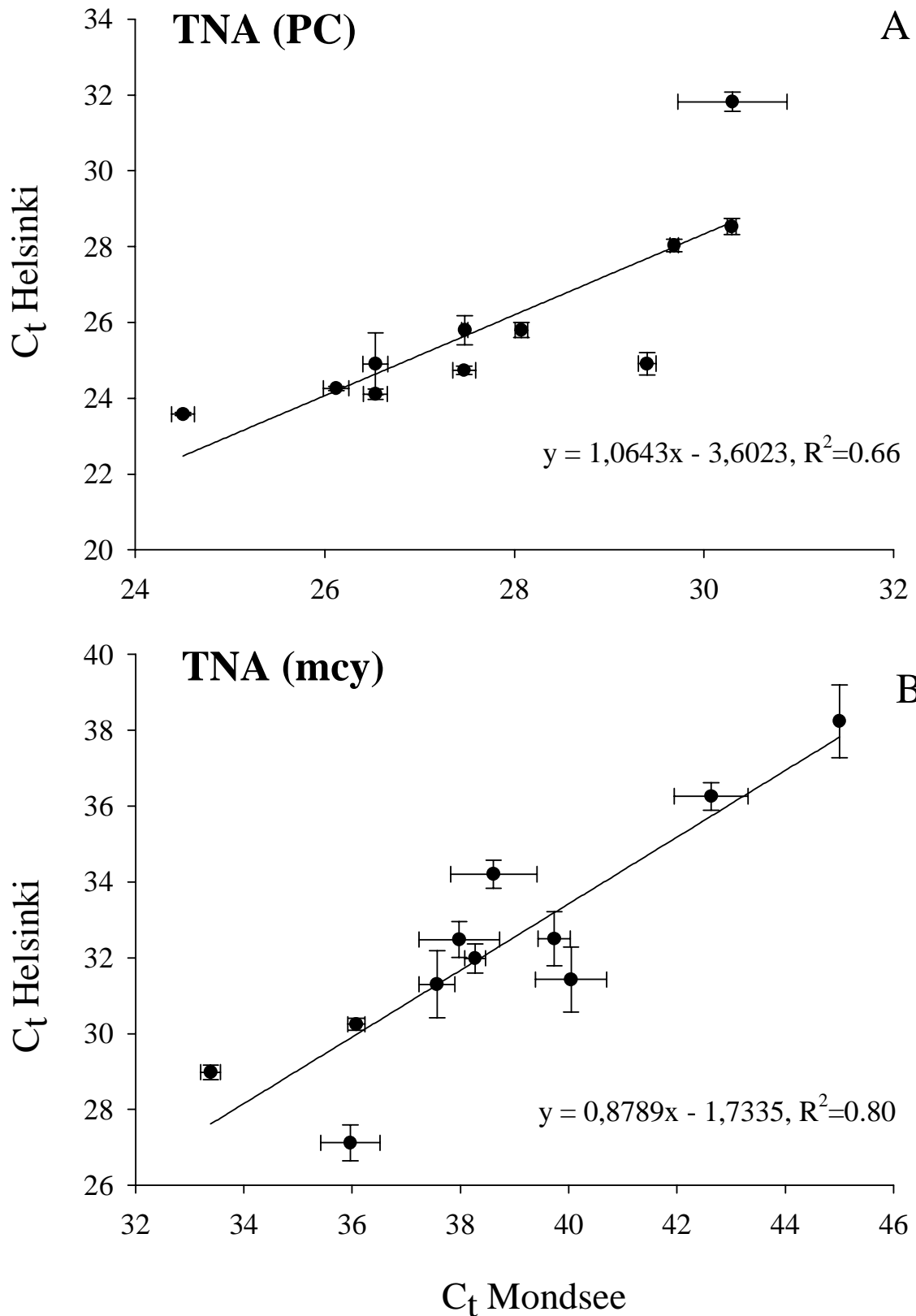


Fig. 7: Threshold cycle values (C_t) obtained by TNA for the phycocyanin gene (PC, mean \pm 1 SE) and the mcyB gene (mcy, mean \pm 1 SE) determined in two laboratories, Mondsee (M) and Helsinki (F). Samples 125, 114, 112, 116 were omitted from analysis (see Tab. 8).

9) Quantitative DNA extraction

Alternatively DNeasy Plant Mini Kit (Qiagen, Cat. No. 69106) can be used for DNA extraction. In order to compare the DNA extraction efficiency between both methods 10 strains of *Microcystis* and 10 strains of *Planktothrix* were harvested, extracted for DNA and cell numbers were quantified using real-time PCR. Additionally different dilutions of one *Microcystis aeruginosa* (HUB 53, Wannsee, Berlin, D) and one *Planktothrix rubescens* culture (number 75, Zürichsee, CH) were filtered and extracted.

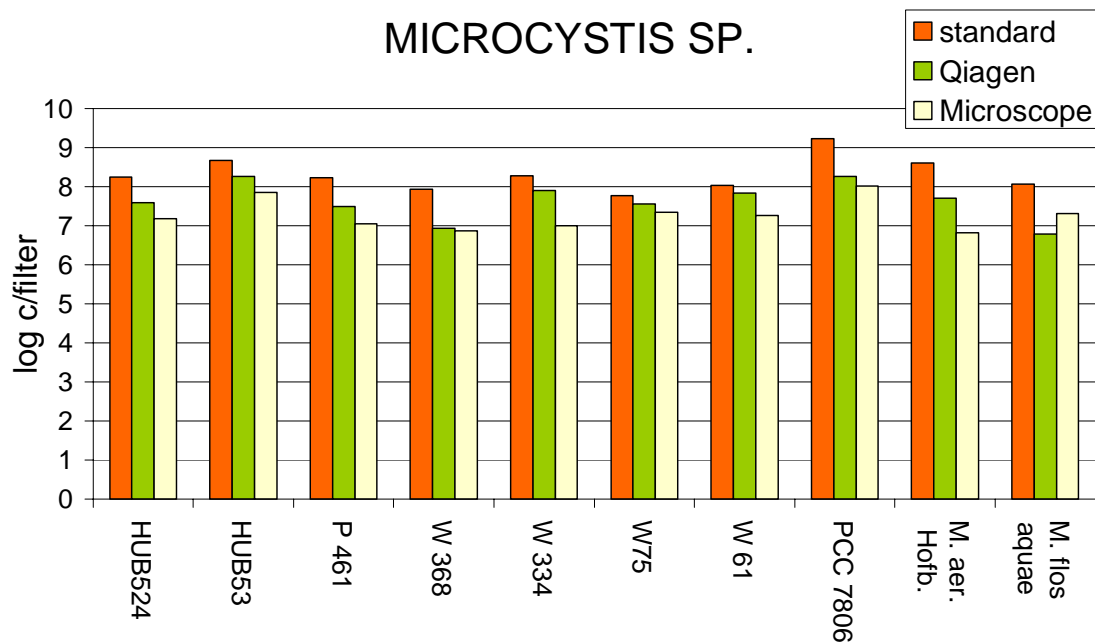


Fig. 8: Quantification of standard and Qiagen DNA extracts from 10 different *Microcystis* strains using rtPCR (MaPC Probe, Phycocyanin generegion).

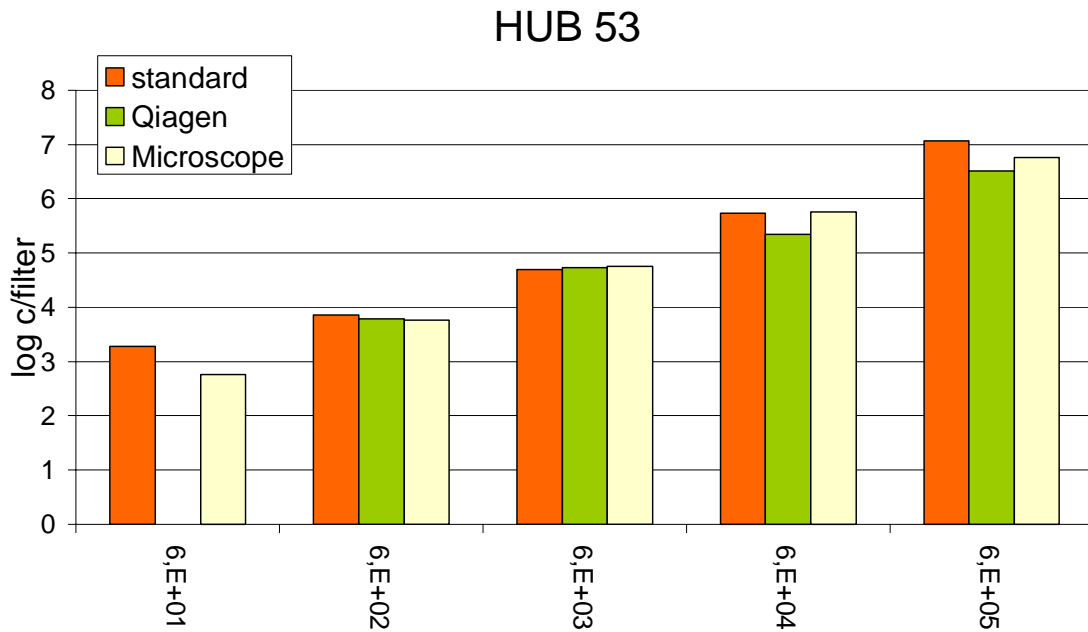


Fig. 9: Quantification of standard and Qiagen DNA extracts from different dilutions of Microcystis strain HUB 53 (Wannsee, Berlin, D).

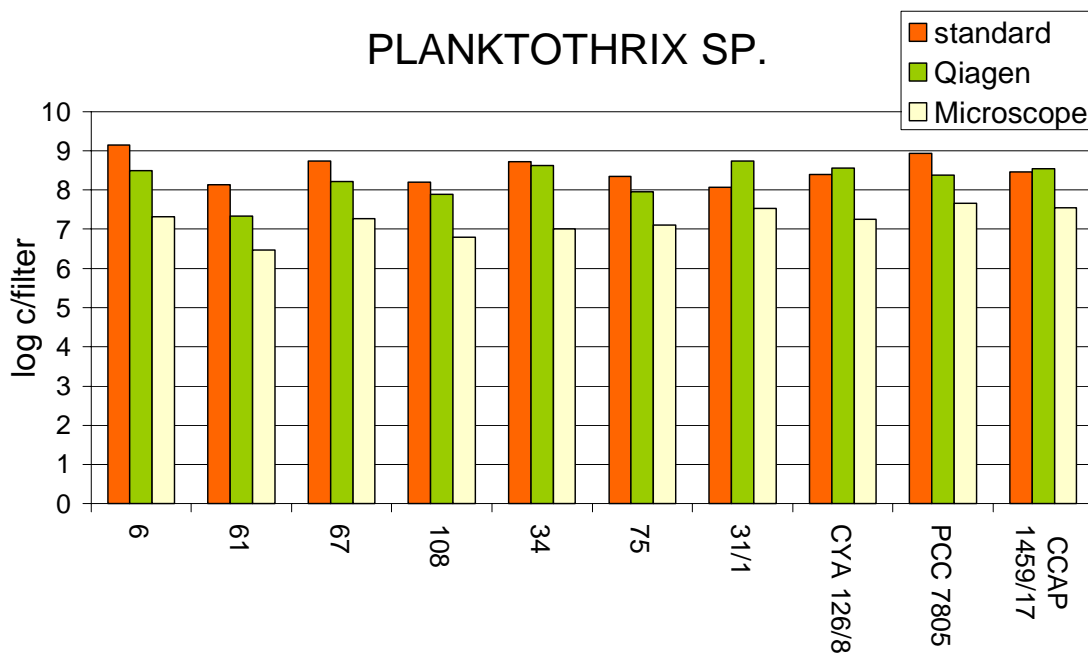


Fig. 10: Quantification of standard and qiagen DNA extracts from 10 different Planktothrix strains using rtPCR (PIPc Probe, Phycocyanin generegion).

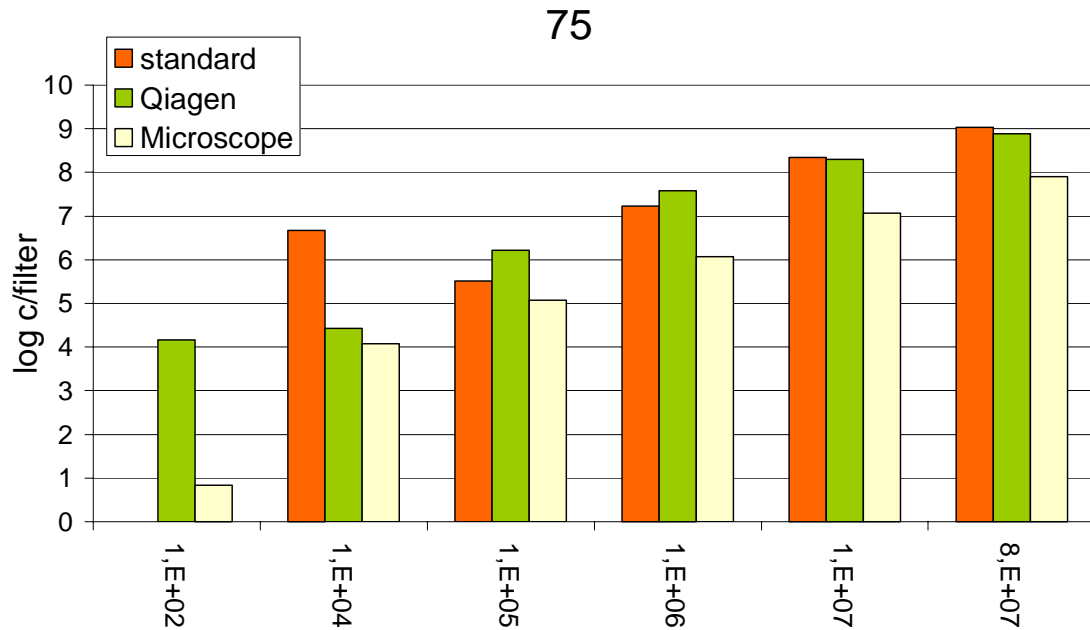


Fig. 11: Quantification of standard and qiagen DNA extracts from different dilutions of Planktothrix strain number 75 (Zürichsee, D).

The results of several experiments comparing standard and Qiagen extraction show, that when comparing the DNA yield (equivalent to cells per ml filtered) the difference in sensitivity between both methods is negligible.

10) Relationship between genotype and chemotype

From the results of physiological work with laboratory cultures microcystin has been interpreted as being a cellular constituent, i.e. always present in microcystin-producing genotypes with cellular concentrations being modified by environmental factors usually two- to fourfold (Orr and Jones 1998, Long et al. 2001, Hesse & Kohl 2001, Böttcher et al. 2001). In contrast a small number of *Microcystis* strains have been repeatedly shown to contain the gene but lack detectable microcystins (Nishizawa et al., 1999; Kaebernick et al., 2001, Tillett al. 2001, Mikalsen et al. 2003). The reason why those strains do not synthesise microcystins is unclear but it has been speculated that mutations within the gene cluster might have occurred in culture (Kaebernick et al., 2001). Results from field populations comparing the occurrence of *mcy* genes with the occurrence of microcystin in individual colonies currently exist only from one lake, i.e. Wannsee (Berlin, Germany), where 28 of 29

(97%) of the colonies found to contain the *mcyB* gene also contained microcystins as shown by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (Kurmayer et al., 2002). In addition, 322 individual colonies sampled from numerous water bodies in Europe were tested for *mcyB* gene distribution and microcystin net production by sensitive matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in parallel and only three individual colonies (1%) were found to contain *mcyB*, but failed to show any detectable microcystin (L. Via-Ordorika et al., unpublished data).

For the population of Lake Wannsee a close relationship between the occurrence of *mcyB* and microcystin net production has been observed (Kurmayer et al. 2003) and a significant relationship between the population growth rate and the microcystin net production rate for the same population in the summer of 2000 has been reported (Kurmayer et al. 2003). In the same study a significant relationship between the population growth rate and the microcystin net production rate for the same population in the summer of 2000 has been reported. From other authors, significant correlations between surrogate parameters such as chlorophyll *a* or algal biovolume and microcystin net production of *Microcystis* sp. have been reported as well (Chorus et al. 2001, Kotak et al. 1995, 2000, Oh et al. 2001). Taken together those results support the conclusion that it is possible to infer microcystin concentrations from surrogate parameters, for example *Microcystis* cell numbers.

From the genetic analysis of 234 filaments analyzed directly from populations of the red pigmented *Planktothrix rubescens* from Lake Ammersee, DE (20 filaments), Lake Irrsee, AT (51), Lake Mondsee, AT (74), Lake Schwarzensee, AT (39), Lake Wörthersee, AT (30) and Lake Zürich, CH (20) 187 filaments contained PC-IGS and all showed the *mcyA* signal (Kurmayer unpublished). The high frequency of occurrence of *mcyA* in red-pigmented populations is in agreement with the field survey by Fastner et al. (1999) who showed that populations of *P. rubescens* had the highest microcystin content when compared to phytoplankton dominated by green-pigmented populations of *Planktothrix* and *Microcystis*.

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