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Distribution and Abundance of Nontoxic Mutants of Cyanobacteria in Lakes of the Alps

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Abstract The filamentous cyanobacterium *Planktothrix rubescens* frequently occurs in deep and stratified lakes in the temperate region of the northern hemisphere and is a known producer of the hepatotoxic secondary metabolite microcystin. These cyclic heptapeptides are synthesized nonribosomally via large enzyme complexes encoded by the microcystin (*mcy*) synthetase gene cluster. The occurrence of cyanobacterial strains lacking microcystin, but containing the *mcy* gene cluster has been reported repeatedly; it was shown that this inactivation is due to mutations such as gene deletion events and the insertion of transposable elements. In the present study, 12 lakes in Austria, Germany, and Switzerland were sampled from July 2005 to October 2007, and the proportion of inactive *mcy* genotypes was quantified in relation to the total population of the red-pigmented filamentous cyanobacterium *Planktothrix* by means of quantitative polymerase chain reaction. In total, four different mutations were quantified, namely two insertions affecting *mcy*D, one insertion affecting *mcy*A, and a deletion within *mcy*H and *mcy*A. The mutations occurred over a wide range of population densities (40–570,000 filaments L^{-1}), and their abundance was found to be positively correlated with population density. However, on average, all nontoxic mutants were found in a low proportion only (min 0%, mean 6.5%±1.1 (SE), max 52% of the total population). The genotype containing the *mcy*HA deletion had a significantly higher proportion (min 0%, mean 3.7%±1, max 52%) when compared with all the genotypes containing insertions within the *mcy* gene cluster (min 0%, mean 2.8%±0.7, max 24%). The results demonstrate that the occurrence of inactive *mcy* genotypes is linearly related to the population density, and selective sweeps of nontoxic mutants did not occur during the transition from prebloom to bloom conditions.

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Introduction

Mass occurrences of bloom-forming toxic cyanobacteria in freshwater are observed worldwide. The filamentous cyanobacterium *Planktothrix* spp. occurs in the pelagial of lakes and reservoirs and is one of the producers of the toxic heptapeptide microcystin that poses a serious health threat to humans and livestock [4]. These hepatotoxins are synthesized by nonribosomal peptide synthetases (NRPS) via the thio-template mechanism encoded by 9–10 genes constituting the microcystin synthetase (*mcy*) gene cluster [6, 33, 38]. In the following, strains of various cyanobacteria have been repeatedly reported to contain the *mcy* genes but lack detectable microcystin [13, 16, 21, 24, 39]. For *Planktothrix*, it has been observed that the *mcy* gene cluster has been frequently inactivated by various mutations, such as insertions of a transposable element or deletions affecting one or two genes of the *mcy* gene cluster [6]. Recently, we were able to show that those mutations, although they may have arisen independently, are of a relatively recent origin, and those nontoxic mutants cannot be discriminated from the strains still containing the intact *mcy* gene cluster by using additional variable genetic markers [7].

Although the poisoning of aquatic grazers via microcystin has been reported [18, 30], it is unlikely that the

microcystins evolved in response to grazing by herbivorous crustaceans. Analyses comparing the phylogenetic trees obtained from housekeeping genes (16S rDNA, *rpo*C1) and *mcy*A, *mcy*D, *mcy*E revealed a congruent branching suggesting that the evolution of microcystin preceded the first appearance of metazoans, probably by one billion years [28]. However, since toxic effects to other aquatic biota cannot be excluded, it might be argued that a specific proportion of microcystin producers in a population is necessary to enable the survival of the individual (nontoxic) cell. Indeed, if these nontoxic mutants can be "cheaters," as in the style of *Myxococcus* sp. strains that have given up various signaling responses [40], one might expect an over representation of nontoxic mutants under bloom conditions relative to their initial frequency at the beginning of the population development. Alternatively, light-limiting conditions as found under dense-bloom conditions have been postulated to result in the gradual increase of nontoxic strains at the expense of toxic strains of the genus *Microcystis* [14]. It has been shown that those inactive *mcy* genotypes occur under natural conditions for several years [6]. However, it is not clear whether those nontoxic mutants are able to dominate the population under bloom conditions.

It was the aim of the present study to investigate the ecological success of the nontoxic mutants in populations of *Planktothrix* that vary in population density. The populations that were studied occurred in 12 lakes in the Alps of Austria, Switzerland, and Germany. Due to the stable physical stratification in deep lakes, the red pigmented *Planktothrix rubescens* dominates at greater water depths with higher amounts of green light when compared with the light conditions at the water surface [44]. *P. rubescens* isolated from Lake Zürich was neutrally buoyant in cultures given 6.5 μmol m^{-2} s^{-1} in a 12-h light cycle and typically *P. rubescens* occurs at depths of 9–12 m with low light intensity [44]. Some of the lakes have been repeatedly reported to show *Planktothrix* blooms (Hallwilersee, Wörthersee, Zürichsee, [12]). Mass developments of the red-pigmented cyanobacteria assigned to *P. rubescens* have also been reported from North America [8, 25], and the occurrence of cyanobacteria assigned to *P. rubescens* has also been reported from New Zealand [27]. We hypothesized that under conditions of prebloom development and sparse population densities, nonmicrocystin-producing mutants should not be able to occur. *Vice versa*, under conditions allowing blooms to occur nonmicrocystin-producing mutants may be able to flourish and gain selective advantage due to a higher growth rate compared to microcystin producers. The quantitative real-time polymerase chain reaction (PCR) technique was used to quantify various inactive *mcy* genotypes and to estimate their share of the total population as described previously [19].

Methods

Study Area and Sampling

Twelve lakes located in the Alps in Austria, Switzerland, and Germany were sampled from summer 2005 to summer 2007. All the lakes are generally deep and physically stratified (Table 1). The trophic state varied from oligotrophic (Attersee, Offensee, Schwarzensee, and Wolfgangsee), and oligomesotrophic (Irrsee, Mondsee, Ammersee, and Fuschlsee), to mesotrophic (Wörthersee, Zürichsee, and Afritzersee), and meso-eutrophic (Hallwilersee). The water column was integrated by collecting 1 L of water every 2 m from the surface to a depth of 20 m. Chlorophyll a and total phosphorus were analyzed according to standard methods [11, 47]. For analyzing the phytoplankton community, aliquots (100 mL) of the samples were preserved with Lugol's solution and 2% formaldehyde each. Additionally, net samples were obtained by pulling a phytoplankton net (30 μm in mesh size) three times from a depth of 20 m to the surface, and aliquots were preserved with a final concentration of 2% formaldehyde. For subsequent DNA isolation, aliquots (2–4 L of integrated samples and 20–100 mL of net samples) were filtered onto glass fiber filters (BMC, Ederol, Vienna, Austria) under vacuum pressure and stored frozen (−20 °C).

Cultivation of Strains and Cell Harvesting

The microcystin-producing *P. rubescens* strain PCC7821 (L. Gjersjøen, Norway) and the inactive microcystin genotypes of *P. rubescens* strains Nos. 110 and 40 (Mondsee, Austria), No. 139 (Grabensee, Austria), and No. 62 (Irrsee, Austria) were grown at 20 °C in BG_{11} medium [29] under constant light conditions (5–15 μmol m^{-2} s^{-1}, Osram Type L30W/77Fluora) and harvested in the exponential growth phase using vacuum filtration onto glass fiber filters (BMC, Ederol, Vienna, AT). Filters were stored frozen (−20 °C) until DNA extraction. Aliquots of the cultures were preserved by adding formaldehyde (final concentration 2%). DNA was extracted and used to relate a series of DNA dilutions (in biovolume equivalents) to the cycle of threshold (C_t value) as measured by real-time PCR.

Cell Counting

Filaments were assigned to the genus *Planktothrix* according to the morphological criteria characteristic for *Planktothrix*

Table 1 List of the study lakes in the Alps and their morphometric characters. Concentrations of chlorophyll a, total phosphorus and secchi depths were averaged (mean ± SE) over the sampling period and used to classify the trophic state [42]

Lake	Country	Latitude	Longitude	Surface area [km^2]	Maximum depth [m]	Number of samples	Sampling period	Trophy[a]	Chl a (mm^3 L^{-1})	TP (mm^3 L^{-1})	Secchi (m)
Attersee	AT	47°54′ N	13°33′ E	46.2	170.6	7	July 05– July 07	O	0.9±0.2	5.0±1.1	9.7±1.7
Wolfgangsee	AT	47°45′ N	13°25′ E	12.8	114	7	July 05– July 07	O	1.2±0.1	4.9±0.5	7.3±1.4
Schwarzensee	AT	47°45′ N	13°30′ E	0.48	54	7	July 05– July 07	O	0.9±0.1	5.6±0.8	6.0±0.6
Offensee	AT	47°45′ N	13°50′ E	0.55	38	7	July 05– July 07	O	2.2±0.3	5.8±0.6	8.6±0.7
Ammersee	DE	47°59′ N	11°07′ E	46.6	81.1	6	July 05– June 07	O-M	1.5±0.3	9.5±1.3	4.0±0.6
Fuschlsee	AT	47°48′ N	13°16′ E	2.65	67.3	7	Aug. 05– Aug. 07	O-M	2.3±0.3	8.1±0.8	5.0±0.4
Irrsee	AT	47°55′ N	13°18′ E	3.6	32	7	July 05– July 07	O-M	1.7±0.2	8.3±0.3	4.5±0.7
Mondsee	AT	47°49′ N	13°22′ E	13.8	68.3	7	Aug. 05– Sep. 07	O-M	2.7±0.4	10±0.5	4.5±0.5
Wörthersee	AT	46°37′ N	14°07′ E	19.4	85.2	6	July 05– May 07	M	3.9±0.6	13±0.7	4.9±1.3
Afritzersee	AT	46°45′ N	13°46′ E	0.5	22.5	6	Aug. 05– May 07	M	4.5±0.8	19.4±2.7	4.9±0.3
Zürichsee	CH	47°15′ N	08°38′ E	65.5	136	7	Aug. 05– Oct. 07	M	7.1±1.4	20.1±3.8	4.4±0.4
Hallwilersee	CH	47°17′ N	08°12′ E	10.2	47	5	Nov. 05– Aug. 07	M-E	8.5±0.6	17.3±1.4	2.6±0.3

[a] *O* oligotrophic, *O-M* oligo-mesotrophic, *M* mesotrophic, *M-E* meso-eutrophic

spp. [1]. Lugol-fixed samples were counted in sedimentation chambers using the inverted microscope technique [41]. At least three transects per chamber were counted at ×100 magnification to obtain a count of at least 400 specimens in total [47]. To estimate the biovolume of *P. rubescens*, 70 filaments from Offensee were measured in diameter using image analysis (Lucia G, on Intrigue Pro Version 4.51). The biovolume was then calculated by assuming the geometric shape of a cylinder and multiplying the mean area of a cylinder by the filament length. The detection limit of *Planktothrix* in the inverted microscope was one filament per sedimentation chamber (25 mL volume). In order to control for counting errors due to a lack of sedimentation of *P. rubescens* filaments [43], aliquots preserved in 2% formaldehyde were filtered onto polycarbonate filters (0.45 µm, Millipore, Vienna, AT), stained with DAPI, and enumerated using epifluorescence microscopy (Zeiss Axioskop 40). At least two transects per filter at ×200 magnification were counted. A significant linear relationship was obtained between the filaments estimated by the sedimentation chamber and by the epifluorescence method: $y=-0.033+0.9722x$ ($R^2=0.85$, $n=18$, min–max=0–4.6 mm^3 L^{-1}), where y is the biovolume (mm^3 L^{-1}) as determined by the sedimentation method and x is the biovolume (mm^3 L^{-1}) as determined by epifluorescence microscopy.

Quantification of Genotypes

Quantitative DNA extraction from filters was performed using the standard chloroform-phenol procedure as described [15]. The TaqMan assay (TNA) was used to quantify (1) the total population of *Planktothrix* via 16S rDNA and (2) the *mcy*BA1 genotype (the first adenylation domain of the *mcy*B gene) that was indicative of all genotypes containing the *mcy* gene cluster and (3) four mutations (three insertions of a transposable element and one deletion) resulting in the inactivation of microcystin biosynthesis [6]. In addition, the insertion sequence (IS) element that was found to be inserted into the *mcy* gene cluster [6] was amplified. The sequence of the IS element was submitted to the IS finder database and denoted *ISPlrub*1 [35]. All primers and probes were designed during this study from sequences published previously [6].

For the design of the TNA amplifying the 16S rDNA of *Planktothrix* spp. two hundred sequences of cyanobacteria

from sections I–V that were collected in the ARB software [20] were aligned, and the primers and probes were designed to amplify *Planktothrix* only. For the amplified region (82 bp), no sequence variation was observed among the 92 entries of the Genbank database (7 April 2008) assigned to *Planktothrix* spp. according to Suda et al. [37]. To quantify microcystin genotypes, a conserved region within the *mcy*B gene, based on 27 strains that were sequenced for the first adenylation domain as described (AJ890255–AJ890282 [17]), was selected. To design the TNAs amplifying specifically the insertions within the *mcy* gene cluster, primers and probes were located both in the insertion and the flanking *mcy* gene region (Fig. 1), i.e., for the 1.4 kbp insertions found in the *mcy*D gene (*mcy*DIS1, strain No. 110 and *mcy*DIS2, strain No. 139) and in the *mcy*A gene (*mcy*AIS, strain No. 40). Analogously, the primers and probes to detect the deletion were designed to bind upstream and downstream of the deleted *mcy* region, i.e., for the 1.8 kbp deletion located within *mcy*H and *mcy*A (*mcy*HA, strain No. 62). For the TNA amplifying the IS element, both primers and probes were located within the IS element.

The primers and probes were designed using the Primer Express 2.0 software (ABI, Table 2). The probes were labeled with a fluorescent reporter dye that was covalently attached to the 5′ end (FAM, 6-carboxyfluorescein) and a fluorescent quencher dye attached to the 3′ end (TAMRA, 6-carboxytetramethylrhodamine). Concentrations of the primers and probes were optimized according to the instruction manual (ABI TaqMan Universal PCR MasterMix). The specificity and robustness of each TNA was tested by adding DNA originating from other organisms as a background. DNA extracted from the *Microcystis* strains HUB524 and HUB53 was added to the DNA of strain No. 62 (*mcy*HA deletion, DNA concentration equivalent to 460 cells per template) and mixed at ratios of 1:100 and 1:1 (calculated in cell equivalents). To test the specificity of the TNA, which was targeted to 16S rDNA and the IS element, DNA extracts from several field samples that were found to be negative by another TNA amplifying the intergenic spacer region of the phycocyanin operon (PC-IGS) of *Planktothrix* spp. [33] were tested. Those samples originated from Austria (Längsee, Höllerersee, and Wolfgangsee in August 2006) and contained the DNA of various other cyanobacteria (*Anabaena* spp., *Lyngbya* spp., *Synechococcus* spp.). No background experiments were performed for the TNAs amplifying the insertions in *mcy*D and *mcy*A, as the design of these TNAs was considered highly specific.

PCR reactions were initiated by a 10-min hold at 95 °C to activate the hot start polymerase, followed by 50 cycles of a two-step PCR, consisting of a denaturation step at 95 °C (15 s) and subsequent annealing and elongation steps at 55 and 60 °C, respectively (1 min each). Each measurement was performed in triplicate using the Eppendorf Mastercycler ep realplex system (Eppendorf, Vienna). The 25-µl reaction mix consisted of 12.5 µl TaqMan Universal PCR Master Mix (ABI), 5 µl of DNA template, and variable concentrations of primers and probes (Table 2). To establish the calibration curves, a dilution series of predetermined DNA concentrations from the extracts of strains was prepared and the DNA content in the template (expressed in equivalents of biovolume) was related to the C_t value (defined as the threshold cycle to reach a manually set fluorescence of 100).

Determination of the Lower Detection Limit

Dilution series of purified PCR products obtained from 16S rDNA, *mcy*BA1, insertions of strains Nos. 110 and 139 ranging from 0.1 to 0.1×10^{-8} fmol were measured by real time PCR as described [34]. The range in dilution corresponded to $3 \times 10^{8} - 0.3$ copies template^{-1}. For each of the target regions, specific C_t values corresponding to 1 copy template^{-1} were determined and used to define a lower limit of detection.

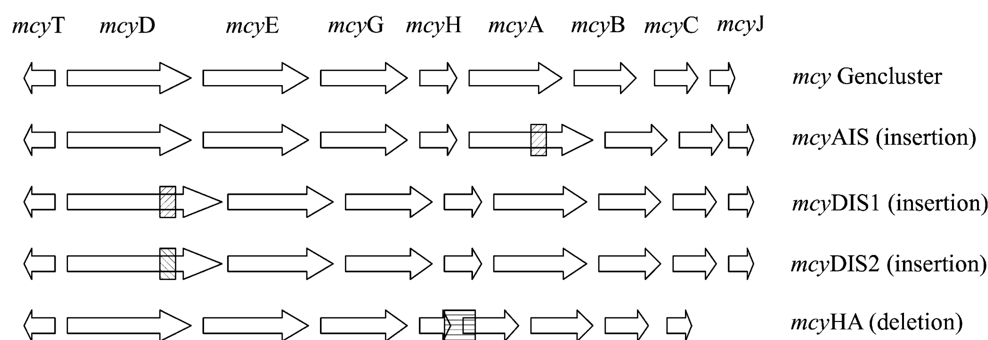

Figure 1 Microcystin synthetase (*mcy*) gene cluster of *Planktothrix* and the location of mutations resulting in the inactivation of microcystin biosynthesis. *Mcy*AIS, *mcy*DIS1, *mcy*DIS2, insertions caused by a transposable (IS) element into genes *mcy*A and *mcy*D of the *mcy* gene cluster, which are transcribed in both directions; *mcy*HA, deletion of parts of the *mcy*H and *Mcy*A genes [6]

Table 2 Oligonucleotide primers and TaqMan probes used for the quantification of the total *Planktothrix* population, the *mcy*BA1 genotype, four inactive *mcy* genotypes and the transposable (IS) element reported to insert into the *mcy* gene cluster [6]

TNA	Gene locus	Strain	Forward primer/Reverse primer (5′–3′)	Taqman Probe (5′–3′)	Concentration (fmol μl^{-1})[a]	Annealing T (°C)	Amplicon (bp)
16S rDNA	16S rDNA	PCC7821	ATCCAAGTCTGCTGTTAAAGA / CTCTGCCCCTACTACACTCTAG	AAAGGCAGTGGAAACTGGAAG	300/300/250	55	82
*mcy*BA1	*mcy*BA1	PCC7821	ATTGCCGTTATCTCAAGCGAG / TGCTGAAAAAACTGCTGCATTAA	TTTTTGTGGAGGTGAAGCTCTTTCCTCTGA	900/900/100	60	76
*mcy*DIS1	3′end IS-Element, *mcy*D	No. 110	TTCTTTACTCTTTTCCACCCGACTT / ACAAATTGCTGTTTTTGCGCT	CGGGAATAGCCCCCCCAAACC	200/200/100	60	93
*mcy*DIS2	3′end IS-Element, *mcy*D	No. 139	TTGAGAATTATGACCCAAAAGTAGGC / ACAAATTGCTGTTTTTGCGCT	CGGGAATAGCCCCCCCAAACC	200/200/100	60	110
*mcy*AIS	3′end IS-Element, *mcy*A	No. 40	TTCTTTACTCTTTTCCACCCGACTT / GAATGAGAGGTAACGGCATTACG	TGACCAGGGCTGGTTTAGCCAATAGTACA	200/200/100	60	168
*mcy*HA	*mcy*HA deletion	No. 62	TCTTCTGGACGGTTTTCTAG / CTTT1CCGGGTTTGATGT	TACAGAATGGGAAAAAATTACTCAAGAGAA	200/200/250	55	71
TIB-TM	IS-Element	PCC7821	ATAGGAGGTATTATCTAAGCAGCAT / GAGGGAAGAAGGTGGTTAGGA	TCCCTATGGAGTAAGACTTACATTCCCGAT	200/200/250	60	176

[a] Concentrations of the forward primer/reverse primer/TaqMan probe

Analysis of the TNA Results

Fluorescence signals below the limit of detection were set to zero. In general, calibration curves were not extrapolated beyond the highest dilution, which was defined arbitrarily as the limit of quantification corresponding to 18 cells $template^{-1}$ for *mcy*DIS2 (strain No. 139) and 4 cells $template^{-1}$ for all the other TNAs. The TNA results between zero and the quantification limit were adjusted to the corresponding quantification threshold. Proportions of all the mutations and the IS element were calculated from the cell numbers (biovolume) detected via 16S rDNA-TNA in the same DNA extract. All linear regression curves were fitted using the least square approximation and the associated statistical tests of Sigma Plot 2000 (V 6.10). The data were $\log_{10}$ transformed in order to achieve normal distribution and constant variances. The linear regressions between the total population density (as estimated from 16S rDNA) and the abundance of the *mcy*BA1 genotype and the *mcy* insertion/deletion mutants (Fig. 5) were compared in slope and intercept using a general factorial model of analysis of variance (ANOVA). The data were modeled as $y = \mu + \beta x + \varepsilon$, where y is the measured abundance of the *mcy*B genotype or a specific *mcy* mutant, μ is the overall mean level, β is the effect of the *mcy*B or *mcy* mutant genotype, x is the effect of the cells of the total population (as determined from 16S rDNA) as a covariate, and ε is the random deviation $N(0, \sigma^2)$ [36]. A SPSS statistical package (V 15.0 for Windows) was used for the ANOVA.

Analysis of Microcystins and Related Peptides in Single Filaments

In order to find out whether nontoxic mutants differ in the production of other related peptides when compared with their microcystin-producing congeners, (109–129 single *Planktothrix* filaments from each lake (a total of 1,230 filaments) were isolated between June 2005 and January 2006. The filaments were isolated under the microscope and analyzed by means of sensitive matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) as described [16]. The automated measurements were performed by Anagnostec GmbH on a Voyager DE Pro workstation (Applied Biosystems) [16, 46]. The following peptides were identified by their molecular mass in accordance with previous studies: Microcystin–LR (molecular weight 995 $[M+H]^+$), desmethyl–microcystin–RR (1,024 $[M+H]^+$), desmethyl–microcystin–LR (981 $[M+H]^+$), microcystin–HtyR (1045 $[M+H]^+$), anabaenopeptin B (837 $[M+H]^+$), anabaenopeptin A (844 $[M+H]^+$), anabaenopeptin E/F (851 $[M+H]^+$), oscillamide Y (858 $[M+H]^+$), oscillapeptin 1088 (1,088 $[M+H]^+$), Cl-aeruginoside 126A (749 $[M+H]^+$), aeruginoside 126A (715

$[M+H]^+$), aeruginosin A (617 $[M+H]^+$), aeruginoside 126B (691 $[M+H]^+$), and aeruginosin 583 (583 $[M+H]^+$) [9, 31, 45, 46]). The absence of the peptides anabaenopeptin B, anabaenopeptin A, and anabaenopeptin E/F was used to identify those filaments (5%) that dropped out from the analysis due to unknown factors.

Results

Efficiency and Specificity of the TaqMan Assays

All of the calibration curves that were established by relating the measured C_t values of DNA extracts to the predetermined DNA concentrations in the template (expressed as biovolume equivalents) showed highly significant linear relations and similar amplification efficiencies (Table 3). The DNA mixture of the *Planktothrix* strain No. 62 (460 cells template^{-1}) and *Microcystis* strains HUB524/HUB53 in a ratio 1:100 revealed a C_t value of 28.55±0.05 (SD), while a hundredfold increase of *Microcystis* resulted in a C_t value of 28.14±0.19. The same DNA concentration of strain No. 62 in the absence of *Microcystis* DNA had a C_t value of 28.37±0.12, and it was concluded that the TNA targeted to the *mcy*HA deletion was specific. In none of the DNA extracts from the field samples that were found to be free of *Planktothrix* in the microscope and by the TNA targeted to PC-IGS, the TNAs for 16S rDNA and the IS element revealed amplification that exceeded the fluorescence threshold during 50 cycles. Consequently, both TNAs were considered specific and robust enough to prevent false positive results.

In total, 79 samples were analyzed. Except of ten samples, *Planktothrix* filaments were detected in all the samples during the counting in the microscope. All the samples except of three (Wolfgangsee, 2006 and two samples from Attersee, 2007) gave positive signals for 16S rDNA, indicating the presence of *Planktothrix*. Comparison of the biovolume estimated using the TNA for 16S rDNA and the biovolume as determined from the microscope revealed a linear relationship following the equation $y=-0.46+0.86x$ ($R^2=0.85$, $n=68$), where y is the $\log_{10}$ biovolume (mm^3 L^{-1}) as determined by the TNA and x is the $\log_{10}$ biovolume as determined by the counting in the microscope (Electronic Supplementary Material, Fig. S1).

Phytoplankton Composition in the Study Lakes

In general, the phytoplankton was composed of species of cyanobacteria, bacillariophyceae, chlorophyceae, cryptophyceae, chrysophyceae, and dinophyceae. Cyanobacteria (*Planktothrix* spp., *Anabaena* spp., *Lyngbya* spp.) and bacillariophyceae (*Fragilaria* spp., *Asterionella formosa*, and various centric diatoms) were the most abundant groups followed by chrysophyceae (*Dinobryon* spp.) and cryptophyceae (*Cryptomonas* spp.). The *Planktothrix* populations studied revealed a wide range of population sizes (40–570,000 filaments L^{-1} corresponding to 3.8×10^3–$5.\times10^7$ cells L^{-1}). According to this variation, the lakes were classified as lakes containing a sparse population of *Planktothrix* sp. only (Attersee, Wolfgangsee, and Schwarzensee), an intermediate population density (Offensee, Ammersee, Fuschlsee, Mondsee, and Irrsee), and a high population density (Afritzersee, Zürichsee, Wörthersee, and Hallwilersee; Fig. 2). On a relative scale, *Planktothrix* constituted <10% of phytoplankton biovolume in lakes with a sparse population only (Attersee, Wolfgangsee, and Schwarzensee) and 50–100% in lakes showing the highest population density (Afritzersee, Hallwilersee, Wörthersee, Zürichsee). In the other lakes, the biovolume proportions were found to be more variable, but *Planktothrix* never constituted >50% of the phytoplankton.

Table 3 Linear calibration curves of TNA used to quantify the total *Planktothrix* population, the *mcy*BA1 genotype, four inactive *mcy* genotypes, and the transposable (IS) element reported to insert into the *mcy* gene cluster [6]

TNA	Gene locus	Strain	Calibration curve[a]	E (%)[b]	R^2	Parameter (*N*)
16S	16S rDNA	PCC7821	$y=36.9-3.836x$	82.3	0.998	11
*mcy*BA1	*mcy*BA1	PCC7821	$y=36.204-3.366x$	98.2	0.998	10
*mcy*DIS1	3′end IS-Element, *mcy*D	No. 110	$y=36.189-3.891x$	80.7	0.997	12
*mcy*DIS2	3′end IS-Element, *mcy*D	No. 139	$y=35.68-3.331x$	99.6	0.988	12
*mcy*AIS	3′end IS-Element, *mcy*A	No. 40	$y=36.73-3.378x$	97.7	0.993	9
*mcy*HA	*mcy*HA deletion	No. 62	$y=39.927-3.677x$	87.1	0.985	9
TIB-TM	IS-Element	PCC7821	$y=36.398-3.83x$	82.4	0.987	11

[a] $y = C_t$ value (PCR cycle number at the fluorescence threshold of 100), x = amount of template DNA (expressed as $\log_{10}$ of cell number equivalents)

[b] Amplification efficiencies (E) were calculated as follows $E=(10^{-1/\text{slope}}-1)\times100$

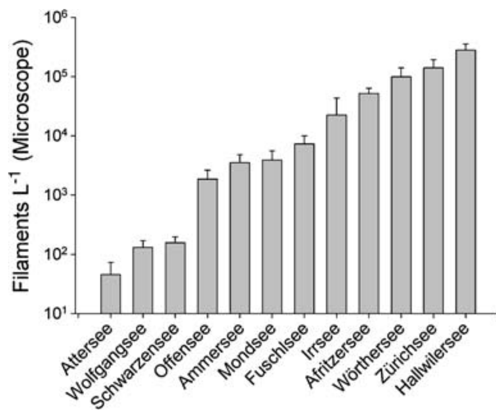

Figure 2 Mean (± SE) filament densities from July 2005 to October 2007 in the 12 study lakes as estimated from the inverted microscope technique

Quantification of Mutants Containing an Inactivated Microcystin Synthetase Gene Cluster

In four of the 12 investigated lakes, all the mutants were detected (Afritzersee, Mondsee, Wörthersee, Zürichsee). In ten out of the 12 investigated lakes, at least one inactive genotype was detected in the integrated lake water samples. In some populations, only specific mutations could be detected, i.e., populations from Offensee and Irrsee contained the *mcy*DIS2 insertion only (Fig. 3). However, the analysis of phytoplankton net samples (resulting in a >1,000-fold concentration of colonial cyanobacteria) revealed the occurrence of other mutations such as the *mcy*HA deletion in Irrsee (data not shown). In contrast, none of the mutants were found in the integrated water samples of Wolfgangsee and Schwarzensee. However, phytoplankton net samples again revealed the occurrence of genotypes containing the *mcy*DIS2 insertion, and it was concluded that the lowest population abundance in Schwarzensee and Wolfgangsee prevented the detection of these mutants in the integrated quantitative lake water samples. It is concluded that nontoxic mutants occurred in all the lakes at all the population densities at least in trace amounts.

Altogether, the *mcy*HA deletion was detected in 47%, *mcy*AIS in 37%, *mcy*DIS1 in 37%, and *mcy*DIS2 in 18% of all the integrated samples. On average, those genotypes containing insertions were found in rather low proportions only (min 0%, mean 2.8%±0.7, max 24%). In contrast, the *mcy*HA deletion was detected more frequently (min 0%, mean 3.7%±1, max 52%). Taking all the data together, the difference in proportions between the insertions and the *mcy*HA deletion was found to be significant (Kruskal–Wallis one way analysis of variance on ranks, $p<0.001$, Fig. 4).

Relationship Between the Population Density and the Abundance of the Mutants

The IS element potentially inactivating microcystin synthesis was detected in all the lakes (77% of the samples were positive). The abundance of the IS element was linearly related to the population density according to the equation $y=-0.69+1.1x$ ($R^2=0.75$, $n=60$), where y is the DNA concentration expressed in $\log_{10}$ biovolume ($mm^3\ L^{-1}$) of the IS element, and x is the DNA expressed in $\log_{10}$ biovolume ($mm^3\ L^{-1}$) of the total population calculated from the 16S rDNA (Electronic Supplementary Material, Fig. S2).

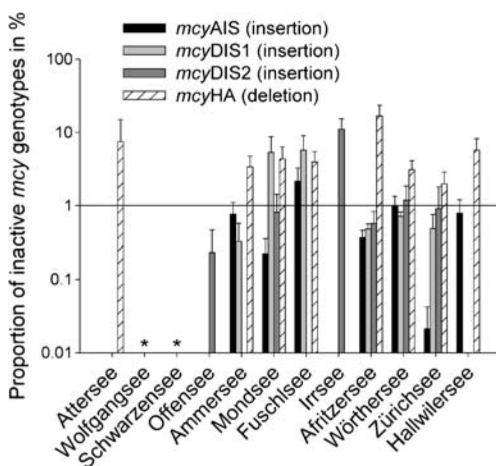

Figure 3 Average (± SE) proportion of inactive *mcy* genotypes in *Planktothrix* populations in the twelve study lakes as quantified by real-time PCR. The *stars* indicate the detection of inactive *mcy* genotypes in the phytoplankton net samples

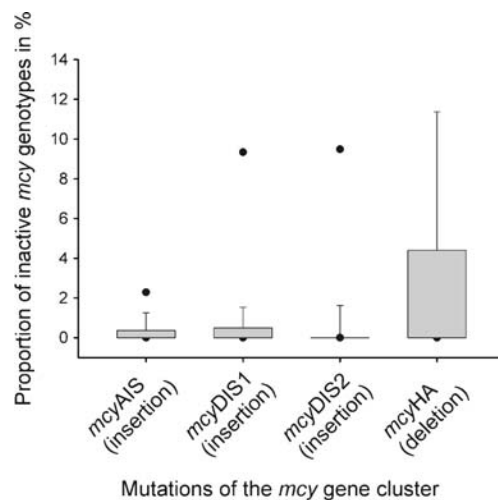

Figure 4 Percentages of inactive *mcy* genotypes in populations of *Planktothrix* ($n=79$). *Box-plots* show the median and the 5–95% percentiles

Correspondingly, the abundance of the *mcy*BA1 genotype was linearly correlated with the population density according to the equation: $y=-0.09+1.14x$ ($R^2=0.96$, $n=76$, $p<0.001$), where y is the $\log_{10}$ biovolume ($mm^3\ L^{-1}$) of the *mcy*BA1 genotype, and x is the $\log_{10}$ biovolume ($mm^3\ L^{-1}$) of the total population. In general, the abundance of the mutations also was linearly related to the population density. The regression equation for the *mcy* genotypes containing insertions was $y=-2.21+0.5x$ ($R^2=0.55$, $n=44$, $p<0.001$), and for the *mcy* genotypes containing the *mcy*HA deletion, the regression curve was $y=-1.49+0.85x$ ($R^2=0.63$, $n=37$, $p<0.001$), where x is the $\log_{10}$ biovolume ($mm^3\ L^{-1}$) of the total population, and y is the $\log_{10}$ biovolume ($mm^3\ L^{-1}$) of genotypes containing an insertion/deletion (Fig. 5). The regression curves calculated for the *mcy*BA1 genotype and the *mcy* mutants containing either the insertions or the *mcy*HA deletion differed significantly in slope (ANOVA, $p\leq0.001$).

Peptide Composition in Single Filaments

In total, microcystin-containing filaments had a share of 72%, while 28% of the filaments were found without microcystin. Besides microcystins, the most abundant peptides detected were the anabaenopeptins B, A, and E/F (95%), and the aeruginosins (55%). The filaments lacking microcystin had a very similar peptide composition when compared with the peptide composition of the microcystin-producing filaments: anabaenopeptin B (98% in filaments without microcystin vs. 99% in filaments with microcystin), anabaenopeptin A (12% vs. 44%), anabaenopeptin F (70% vs. 90%), oscillamide Y (2% vs. 22%), oscillapeptin 1088 (4% vs. 17%), Cl-aeruginoside 126A (17% vs. 21%), aeruginoside 126A (8% vs. 32%), aeruginosin A (8% vs. 22%), aeruginoside 126B (11% vs. 6%), and aeruginosin 583 (18% vs. 12%). None of the peptides occurred with a significantly higher frequency among the microcystin deficient filaments when compared with the microcystin-producing filaments (*t*-test, $p>0.1$).

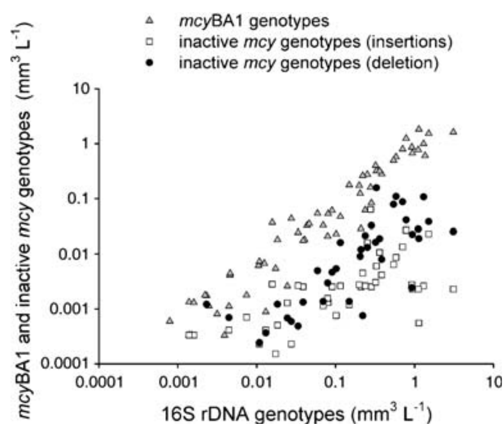

Figure 5 Relationship between the total population density (in biovolume $mm^3\ L^{-1}$) estimated from 16S rDNA and the biovolume estimated from the *mcy*BA1 genotype (*gray triangles*), the sum of inactive *mcy* genotypes containing insertions (*white squares*) and the inactive genotype containing the deletion (*black circles*). For details on the calculation of the respective regression curves, see text

Discussion

Evolution of the Microcystin Synthetase Gene Cluster

In the present study, the abundance of nontoxic microcystin mutants was monitored for 2 years. Although the proportion of the inactive *mcy* genotypes was found to vary during the sampling period, no trend of increase or decrease in the proportion through the study period was observed. Instead, we concluded that the abundance of all the nontoxic mutants was primarily related to the population density. These first quantitative estimates confirm previous results, which reported the regular detection of these mutations through the years 2001–2004 [6]. It is generally anticipated that prokaryotic organisms maintain rather small genomes due to a continuous loss of mutated and, therefore, nonfunctional genetic material [22]. However, because the population numbers are huge, only larger gene deletions are considered to provide sufficient selective advantage so that mutations resulting in gene loss become fixed on a global scale [3]. We recently reported that only larger deletions that lead to the loss of a major part of the *mcy* gene cluster resulted in the distribution of nontoxic mutants across the European continent [7]. However, this was in the course of rather long time periods, probably spanning millions of years. Rohrlack et al. [31] described the co-occurrence of *Planktothrix* strains differing in peptide composition over more than 30 years in Lake Steinsfjorden (Norway) suggesting a rather slow evolution of NRPS in *Planktothrix*. Consequently, successional studies exceeding the usual investigation period of a few years will be needed in order to relate the increase or decrease of specific *mcy* genotypes and their nontoxic mutants to the evolution of the *mcy* gene cluster.

Spatial Isolation of Populations

The results on the average stable proportion of nontoxic mutants are important in order to predict the toxicity of blooms formed by *Planktothrix* in lakes and reservoirs. It is known that *Planktothrix* is one of the more efficient invaders and several examples of the sudden appearance of blooms in Europe have been reported [23, 26]. According to the results of this study, inactive *mcy* genotypes are

unlikely to dominate populations by a selective sweep during the transition from prebloom conditions to bloom conditions in European lakes. Instead, it is proposed that these mutants increase only slowly over the time, and it is not yet possible to predict whether these mutations become fixed on a global scale. One major difficulty to predict their fixation is that populations have been found to diverge in genotype composition due to spatial isolation [17]. In this study, some populations had only one inactive *mcy* genotype, i.e., in Offensee and Schwarzensee, only the *mcy*DIS2 genotype could be detected. This phenomenon is believed to result from the spatial isolation of populations among different lakes leading to a divergence in genetic population structure for several years. Although this isolation may be linked to a relatively stable co-occurrence of genotypes over decades [31], it is not believed that this effect leads to a complete divergence in the evolution of microcystin synthesis, as genetic exchange has been observed both on a scale of a few kilometers [17] as well as a larger scale covering hundreds of kilometers [2].

Selective Pressure on Inactive *mcy* Genotypes

From the data presented (Fig. 5), it is concluded that on average, the abundance of the inactive *mcy* genotypes was found linearly related to the population density. For both *mcy* mutants containing the insertions and the *mcy*HA deletion, the slope of the regression curves was significantly lower when compared with the slope of the regression curve obtained for the *mcy*BA1 genotype. It is emphasized, however, that it would be premature to conclude whether selection may disfavor a single *mcy* mutant or not. If the nontoxic mutants would be selectively disfavored due to the lack of microcystin in general, then this selective disadvantage is considered to be of relatively minor importance when compared to the overall increase and the distribution of the *mcy* mutants among most of the study lakes.

Alternatively it is possible that the mutants investigated during this study differ in the production of a putative yet undetected peptide that compensates for the lack of the microcystin. In this study, the frequency of occurrence as recorded for the most widely distributed anabaenopeptins and aeruginosins did not differ between the two groups. Other more frequent peptides such as cyanopeptolins and microviridins were not considered in this analysis. Consequently, as argued by one reviewer, it is possible that those nontoxic mutants are not “cheaters” but rather follow another strategy by producing other peptides replacing the microcystin. In either case, we found an astonishing constancy of the share of the nontoxic mutants in relation to a >10,000-fold range in population density. This implies that the selective (dis)advantage to the individual due to the production of a putative peptide should be relatively independent from the density of the total population.

Effects of the Deletion or the Insertional Inactivation of the *mcy* Genes on Cellular Growth

In general, the deletion of parts of the *mcy* gene cluster was observed more frequently when compared with the insertions (Fig. 4). Studies on gene loss processes generally suggest a mutational bias for deletions that leads to the erosion of the inactivated genetic material. This is considered to counteract the continuous acquisition of genetic material by horizontal gene transfer [22]. It is not yet known whether even smaller deletions as observed in this study have the potential to increase the growth rate due to a reduction in genome size, in contrast to insertions that result in an increased burden of DNA amplification during cell division. On the other hand, as NRPS are large multifunctional enzyme complexes, high metabolic costs solely due to the microcystin synthesis have been proposed. Surprisingly, the comparsion of the growth of microcystin deficient mutants and that of the wildtype strain of *Microcystis aeruginosa* strain PCC7120 did not reveal significant differences [10]. During the study period, the *mcy*HA deletion was found to have the broadest distribution occurring in lakes that are hundreds of kilometers apart. In the future, comparative growth experiments under controlled conditions in the laboratory between the mutants containing deletions and insertions will need to demonstrate whether the net decrease in gene content can indeed result in higher growth rates.

Distribution of the IS Element *Plrub*1

In this study, the relation between the abundance of the total population and the abundance of the IS element *Plrub*1 reported to inactivate the *mcy* gene cluster was linear. It is concluded that this IS element that inactivated microcystin synthesis in *Planktothrix* is indeed specific for *Planktothrix* spp. and does not occur in other organisms. It is likely that this IS element is active and contributes to a number of mutations affecting other parts of secondary metabolite synthesis. So far, the IS element has been found in all strains assigned to *P. rubescens*, while green-pigmented strains of *P. agardhii* differed significantly in the content of the IS element (C. Molitor, R. Kurmayer, unpublished data). It is possible that this IS element constitutes an important factor currently inducing genetic variation among red-pigmented *Planktothrix* populations in European lakes. It would be an interesting task to find out whether the activity of this IS element can vary and if this may be influenced by specific environmental conditions.

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